THE EXPRESSION AND DETECTION OF *ESCHERICHIA COLI* LIPOPOLYSACCHARIDE WITH MONOCLONAL ANTIBODY PROBES

DAVID NELSON

Thesis presented for the degree of Doctor of Philosophy

University of Edinburgh

1991

CONTENTS

ABBREVIATIONS

Abbreviations frequently used and/or novel to this thesis are listed below.

A	absorbance
BAPS	biotinylated alkaline phosphatase-streptavidin
bio-MAb(s)	biotinylated monoclonal antibody
BSA	bovine serum albumin
DIC	disseminated intravascular coagulation
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
HDLP	high density lipoprotein
HRP	horse-radish peroxidase
IL-1	interleukin-1
IVIG	intravenous immunoglobulin
KDO	3-deoxy-D-manno-2-octulosonate
LAL	*Limulus* amoebocyte-lysate
LBP	lipopolysaccharide binding protein
LOS	lipooligosaccharide
LPS	lipopolysaccharide
MAb(s)	monoclonal antibody
MIC(s)	minimum inhibitory concentration
NeuNAc	N-acetylneuramic acid
NIC	nitrocellulose
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
R-LPS	rough lipopolysaccharide

SAP	streptavidin alkaline phosphatase
SD(s)	standard deviation of the mean
SDS	sodium dodecylsulphate
S-LPS	smooth lipopolysaccharide
TBS	tris buffered saline
TNF	tumour necrosis factor
TTBS	Tween tris buffered saline

ABSTRACT

Lipopolysaccharide (LPS) is a major constituent of the outer membrane of all Gram-negative bacteria and is known to be responsible for the range of pathophysiological features of endotoxic shock. This thesis considers the expression of *Escherichia coli* LPS under different environmental conditions and its detection in the serum of septic patients by means of monoclonal antibody (MAb) probes.

From an existing panel of MAbs, reactive with either Lipid A, core oligosaccharide and O-polysaccharide components of LPS, eleven were selected and characterized in a number of assay systems. Immunoblotting established that core reactive MAbs were reactive against either core not substituted with O-antigen or both substituted and unsubstituted core material. Core-glycolipid reactive MAbs demonstrated either full cross reactivity against all *E. coli* core types, or preferential binding to selective *E. coli* core types. Flow cytometric and ELISA analysis on whole bacteria showed that the absence of O-antigens on rough mutants increased accessibility of core-glycolipid LPS to antibodies. Analysis of the whole cell ELISA technique established that the expression of whole cells on ELISA plates differed from those in suspension.

Sandwich ELISA methods employing suitable combinations of solid phase and biotinylated secondary MAbs were developed for the detection of *E. coli* core types R1-R4, specific core types R1 and R3 and *E. coli* O18, O-antigen. The sensitivity of the assay using the two most cross-reactive MAbs was between 0.01 and 10 ng ml^{-1} *E. coli* LPS, depending on

the core type. The sensitivity of assays for the detection of specific core and O-types was between 0.01 and 0.1 ng ml^{-1} *E. coli* LPS. Assay sensitivity was significantly reduced for the detection of LPS in spiked serum. Methods to improve the sensitivity were investigated.

MAb probes were used to study the expression of LPS on *E. coli* strains grown under a variety of conditions in batch culture which mimicked some of the *in vivo* environmental conditions of an infected host. Growth in heat-inactivated sheep serum and magnesium depleted conditions demonstrated increased expression of LPS core and subsequent increased binding of anti-core MAbs. Iron depleted bacteria also showed increased binding of anti-core MAbs. Nitrogen deficient/high carbon conditions, chosen to promote capsule production, resulted in increased expression of O-antigen and decreased binding of anti-core MAbs. The expression of LPS *in vivo* grown bacteria was investigated using chamber implants and a mucin-haematin septicaemia model. *In vivo* bacteria expressed greater amounts of O-antigen and unsubstituted core material as well as an overall increase in O-antigen chain length. ELISA inhibition studies of anti-core MAb binding established greater inhibitory activity of *in vivo* cells against *in vitro* cells.

The binding capacity of anti-LPS MAbs to *E. coli* bacteria grown in the absence and presence of sub-minimum inhibitory concentrations (sub-MICs) of various antibiotics was studied. Treatment with ampicillin, chloramphenicol and ciprofloxacin resulted in enhanced binding of anti-core reactive MAbs to most *E. coli* strains. Overall, treatment with gentamicin produced the least effect on MAb binding. The presence of chloramphenicol decreased the expression of high molecular mass O-

antigen and/or an increased expression of low molecular mass substituted *E. coli* LPS. The outer membrane proteins of *E. coli* strains grown in the presence of sub-MICs of ciprofloxacin and gentamicin, expressed several additional minor high molecular mass proteins.

The relevance and significance of these studies, in relationship to the patient with septic shock, is discussed.

PUBLICATIONS

1 **NELSON, D., NEILL, W. & POXTON, I.R.** (1990) A comparison of immunoblotting, flow cytometry and ELISA to monitor the binding of anti-lipopolysaccharide monoclonal antibodies. J. Immunol. Methods 133, 227-233.

2 **NELSON, D., NEILL, W. & POXTON, I.R.** (1990) Flow cytometry, ELISA and immunoblotting for monitoring binding of anti-LPS monoclonal antibodies. The First Congress of the International Endotoxin Society, San Diego. Abstract I-P-92.

3 **NELSON, D., BATHGATE, A.J. & POXTON, I.R.** (1991) Monoclonal antibodies as probes for detecting lipopolysaccharide expression on *Escherichia coli* from different growth conditions. J. Gen. Microbiol. (In press).

ACKNOWLEDGEMENTS

During my three years in Edinburgh I have received the help and support of a number of people. The patient guidance, encouragement and understanding of my supervisor, Dr Ian Poxton has been much valued and appreciated. I am also extremely grateful to Dr Robin Barclay for his enthusiasm, help and advice, and for granting intermittent residence in his laboratory at the S.E. Scottish Blood Transfusion Service to yet another Nelson.

Sincere thanks are extended to Robert Brown for his faultless technical advice throughout this study. The cooperation and help of Linda Milne is also appreciated. Bill Neill is thanked for tirelessly and skillfully operating the flow cytometer, whilst the help of Derek Notman and Una Gray with the electron microscopy studies is much appreciated. The contribution of Toby Delahooke to the antibiotic work is gratefully acknowledged. Dr Keith James and colleagues of the Department of Surgery are thanked for performing the fusions and producing the MAb supernates. Sandoz is thanked for financing this work.

I applaud the unrivalled application, commitment and ability of my personal secretary - my mum, throughout the preparation of this written thesis. I also thank Lisa Calderwood and Ian Brown for the photographic components.

Finally, over the years, I have been fortunate to have had unstinting support and encouragement from my mum and dad and brother James, and for this I am lastingly grateful.

DECLARATION

All of the investigations and procedures presented in this thesis were performed by the author unless otherwise indicated in the acknowledgements.

INTRODUCTION

CHAPTER 1

GRAM-NEGATIVE SEPTICAEMIA

1.1 GENERAL FEATURES

Infections by Gram-negative bacteria are a serious and increasing problem, especially those associated with the clinical syndromes of septicaemia and septic shock. The presence of bacteria in the blood, termed bacteraemia, can often be transient and of minimal consequence particularly for immunocompetent individuals. Conversely, bacteraemia may proceed to septicaemia, a condition associated with a systemic response to the presence of microorganisms or their products in the bloodstream or tissues, characterized by several clinical findings (Dudley, 1990). Septicaemia frequently progresses to septic shock, described as peripheral circulatory failure with inadequate tissue perfusion and cell death secondary to an infectious process (Dipiro, 1990). The infectious process resulting from a focus of infection is often referred to as 'sepsis'. One of the first descriptions of Gram-negative bacteraemia dates back to the 19th century (Brill & Libman, 1899). Prior to the advent of antimicrobial agents, Gram-negative septicaemia was a clinical and laboratory curiosity (Young *et al*, 1977). However, the extensive use of antimicrobial agents over the past 30 years has heralded the emergence of Gram-negative opportunist pathogens as an important group of organisms responsible for high morbidity and mortality. Despite the availability of potent antibiotics, the use of vasoactive drugs and intensive care, the mortality rate of septic shock is currently 60-80% (Wardle, 1979; Ziegler *et al*, 1982; Bryan *et al*, 1983; Ispahani *et al*, 1987). Since 1950 the incidence of Gram-negative septicaemia and shock has increased to a

level that it is a frequent complication in the management of hospitalized patients. In the United States the rate has risen from 1 per 1,000 hospital admissions in 1950 to about 13 per 1,000 in 1980 (Kreger *et al*, 1980a). Consequently, septic shock is presently the commonest cause of death in intensive care units in the United States (Wilson, 1984).

Experimental and clinical evidence suggests that endotoxin, primarily the lipopolysaccharide (LPS) component of the Gram-negative bacterial outer membrane, is one of the most important components in the development of severe septicaemia and shock (Ryan, 1985). The terms Gram-negative shock or endotoxic shock are commonly used to reflect the aetiology and the central role played by bacterial LPS.

1.2 AETIOLOGY

Although the causative organisms of septicaemia are many and varied, those caused by aerobic or facultative Gram-negative rod-shaped bacteria predominate, accounting for between 20% and 50% (Dudley, 1990). Since mortality rates from Gram-negative bacteraemia range from 20 to 60% these organisms are recognized as important agents of infection (Kreger *et al*, 1980a; Ispahani *et al*, 1987; Gransden *et al*, 1990).

The organisms responsible for the majority of septicaemic cases have been determined in a number of published studies of septicaemia, both from the United Kingdom (Young, 1982; Ispahani *et al*, 1987; Eykyn *et al*, 1990) and elsewhere (Bruun *et al*, 1982; Weinstein *et al*, 1983; Peltola *et al*, 1987; French *et al*, 1990; Neu *et al*, 1990). Despite inherent variation because of differences in patient population and

hospital practices, the predominance of certain organisms is common to all. The comprehensive septicaemic study of Eykyn *et al* (1990) covered nearly 4,000 episodes at St Thomas' Hospital from 1969-1988. Table 1 summarizes the organisms commonly isolated from both hospital and community acquired septicaemic patients in this report.

Table 1. Significant microbial isolates from septicaemic patients (Eykyn *et al*, 1990)

Percentage of Infections			
Hospital acquired (2,518)		Community acquired (1,750)	
Escherichia coli	20	*Escherichia coli*	25
Staphylococcus aureus	19	*Streptococcus pneumoniae*	22
Klebsiella species	9	'viridans' streptococci	10
Pseudomonas species	9	*Staphylococcus aureus*	10
Coagulase-negative staphylococcus	7	β-haem streptococcus	6
Proteus mirabilis	6	anaerobes	4
anaerobes	5	*Proteus mirabilis*	3
Other enterobacteria	5	*Klebsiella* species	3
Others	20	Others	17

Similar to other published series the most common isolate was *E. coli* (22%). Other members of the Enterobacteriaciae are also well represented, particularly in hospital acquired infection, together with non-enteric *Pseudomonas* species. Despite the predominance of *E. coli*, only eight O-serotypes from a total of over 160 are associated with more than 50% of *E. coli* septicaemias (Kreger *et al*, 1980a; Cross *et al*, 1984). Similarly, *Pseudomonas aeruginosa* (Moody *et al*, 1972; Dick *et*

al, 1988) and *Serratia marcescens* (Gaston *et al*, 1988) both show an association of certain O-serotypes with septicaemia. *S. aureus* is commonly second only to *E. coli* as a cause of septicaemia, accounting for 15%, (Eykyn *et al*, 1990) a figure comparable with the 17.8% reported by Young (1982) and the 11% by Ispahani *et al* (1987).

Septicaemia complicated by septic shock is associated with an increase in mortality rate (Dudley, 1990). Although endotoxin has been strongly implicated in the syndrome of septic shock, not all cases are caused by Gram-negative bacteria (Cohen, 1989). The current view is that other organisms, including Gram-positive bacteria, may also cause a shock-like syndrome indistinguishable from that induced by Gram-negative bacteria (Bayston & Cohen, 1990). Overwhelming *S. aureus* septicaemia, and the pneumococcal septicaemia of splenectomized patients provide good illustrations of serious Gram-positive sepsis associated with shock (Easmon, 1990). Recent evidence indicates that whilst endotoxin is perhaps the most potent mediator of septic shock, it is probably one of several important bacterial products that can produce the disease (Bate *et al*, 1988; Jupin *et al*, 1988; Natanson *et al*, 1989). It appears that regardless of aetiology, a final common pathway is involved in the production of septic shock. Nevertheless endotoxin is likely to be responsible for cases of shock associated with Gram-negative septicaemia.

Bacteraemia is not necessarily a prerequisite for septicaemia which can occur without a positive blood culture. Indeed, Danner *et al* (1991) established that 74% of clinically diagnosed cases of septic shock with detectable endotoxaemia had negative blood cultures, an observation common to many others. Whilst rendering blood cultures negative, host

defences or antibiotics or both, release endotoxin into the circulation as a result of bacterial lysis (Shenep *et al*, 1985a; Cohen & McConnell, 1986; Shenep *et al*, 1988). Additionally, extravascular sources of endotoxin such as the gastrointestinal tract (Ravin *et al*, 1960; van Deventer *et al*, 1988b; Sori *et al*, 1988), or sequestered foci of infection may also be important. If the leakage of endotoxins from the gut exceeds the capacity of the natural de-toxification mechanism, 'non-septic endotoxic shock' may develop (Tamakuma *et al*, 1971; Cuevas & Fine, 1972). Circulating endotoxin has also been detected in some cases of Gram-positive or Candida septicaemia (van Deventer *et al*, 1988a; Danner *et al*, 1991). The source of this endotoxaemia may be undetected Gram-negative bacteraemia, or alternatively release of endotoxin from extravascular sites such as the gastrointestinal tract as a consequence of the primary Gram-positive infection.

1.3 SEPTICAEMIA AND THE DETECTION OF ENDOTOXIN

The most widely used and most sensitive method of detecting endotoxin is the *Limulus* amoebocyte-lysate (LAL) assay (Sturk & ten Cate, 1985; Jorgenson, 1986). This bioassay takes advantage of the fact that a lysate of blood cells (amoebocytes), from the horseshoe crab (*Limulus polyphemus*), gels on exposure to picogram quantities of endotoxin (Cohen, 1989). The relatively recent modification of the original assay by the addition of a chromogenic substrate, has allowed the development of a fully quantitative micro assay (Cohen & McConnell, 1984).

Although the LAL assay is put to many uses, the greatest debate surrounds its use in the diagnosis and management of suspected Gram-negative septicaemia. Initially it seemed as though the presence and

degree of endotoxaemia correlated with outcome (Levin *et al*, 1972). It has become apparent however, that endotoxin is not always present in serum of patients with Gram-negative septicaemia, and that conditions other than sepsis may result in endotoxaemia (Jorgenson, 1986). Thus a positive *Limulus* test is not useful in guiding the selection of antibiotic therapy since it does not predict the presence of Gram-negative sepsis or the absence of Gram-positive or fungal sepsis (Danner *et al*, 1991). The absence of endotoxaemia in septicaemic cases may be due to its rapid clearance from the circulation (Thomas *et al*, 1984; van Deventer *et al*, 1988b); the sequestration of endotoxin at local sites of infection, or that toxins other than endotoxin may be wholly or partially responsible for producing the septic shock syndrome in some patients (Danner *et al*, 1991).

Currently, the most promising use of the LAL assay in septicaemia is a guide to prognosis. Danner *et al* (1991) established that qualitatively, the presence of endotoxaemia was associated with severe septic shock and organ injury. The same study also determined a connection between patients with positive blood culture and endotoxaemia with high mortality. The LAL assay has also been valuable in highlighting the important role of endotoxaemia originating from the gut in the clinical manifestations of liver and gastrointestinal diseases and massive trauma (Tamakuma *et al*, 1971; Nolan, 1975; 1990).

1.4 EPIDEMIOLOGY

A number of underlying reasons account for the high and increasing incidence of septicaemia. Perhaps one of the most important groups of predisposing conditions is the use of invasive medical procedures. Surgical manipulation can cause septicaemia or endotoxaemia as a

consequence of contamination and infection of wounds and exposed surfaces from environmental or endogenous sources (Freeman & Gould, 1985; Nagachinta *et al*, 1987; Rocke *et al*, 1987). The manipulation of the gastrointestinal tract, a rich source of Gram-negative organisms and endotoxin is of particular risk. Two frequently recorded foci of infection in hospital acquired septicaemia include the urinary tract and intravascular access sites, usually from catheterization or instrumentation (Eykyn *et al*, 1990). These urinary tract associated septicaemias are commonly attributed to *E. coli*, whilst the primary focus for staphylococci is infected intravenous access sites. Despite the frequency of *E. coli* bacteraemia, the organism has been reported absent from infected intravenous lines (Eykyn *et al*, 1990; Gransden *et al*, 1990). An observed increase in the prevalence of coagulase negative staphylococci, *S. aureus*, and enterococci by Neu *et al* (1990) was attributable to greater use of indwelling lines, and to better practices in preventing *E. coli* related urinary tract infections by attention to urinary catheters.

Underlying state of health is a well recognized influence on the outcome of Gram-negative bacteraemia (Bryant *et al*, 1971; Kreger *et al*, 1980a). Diseases such as cancer, renal failure, congestive heart failure, and diabetes mellitus are all related to an increase of septic shock. An impaired liver function is also related to the development of septicaemia or endotoxaemia (Triger *et al*, 1978; Gaeta *et al*, 1982; Lumsden *et al*, 1988). Normally, LPS enters the liver continually from the gut and is removed by the liver hepatocytes preventing both the entry and persistence in the circulation (Fox *et al*, 1990). A decreased removal of LPS by hepatocytes correlates with a lower survival rate from septicaemia (Jacob *et al*, 1977; Katz *et al*, 1984).

Immunosuppression induced by cancer treatments or transplantation regimens also increases the risk of septicaemia (Dipiro, 1990), as well as immunodeficient patients with diseases such as neutropenia (Minah *et al*, 1986) and acquired immune deficiency syndrome (AIDS) (Eng *et al*, 1987; Sperber & Schleupner, 1987). Severe burns or multiple trauma also result in a lowering of the immune status allowing colonisation of wounds by organisms such as *Pseudomonas* species (Mason *et al*, 1986; Deitch *et al*, 1987; Winchurch *et al*, 1987). Further high risk groups include neonates and the elderly, their lowered immune status succumbing to septicaemia and meningitis caused by enterobacteria (Eykyn *et al*, 1990).

McCabe & Jackson (1962) proposed a classification system for determining the risk of mortality for Gram-negative bacteraemia according to the severity of the underlying disease. The first category was associated with rapidly fatal disease such as acute leukaemia, whilst the second category represented patients whose disease was ultimately fatal, but whose lives were not in immediate danger, for example, patients undergoing chemotherapy or surgery for gastrointestinal carcinomas. The third category included patients with non-fatal diseases such as diabetes and other chronic illnesses which predisposed them to infection. More recently, Gatell *et al* (1988) devised a scheme in which the variable associated with the highest risk of mortality among patients with hospital acquired (also termed nosocomial) Gram-negative bacteraemia, was shock.

Despite the prevalence of nosocomial septicaemic episodes, a significant number are acquired within the community. Community acquired septicaemias arise spontaneously, often in previously healthy people,

whereas nosocomial infections are largely the result of medical intervention (Eykyn *et al*, 1990). In most reported studies the organisms of *E. coli*, *S. pneumoniae* and *S. aureus* usually predominate community acquired septicaemias, whilst enterobacteria other than *E. coli* and *P. aeruginosa* are usually more common in nosocomial infections (Ispahani *et al*, 1987; Eykyn *et al*, 1990; Neu *et al*, 1990). Many result from infections at a local site, such as pneumococcal pneumonia or acute pyelonephritis, or invasion of the blood, often from an undetected site as in staphylococcal osteomyelitis or endocarditis.

1.5 CLINICAL FEATURES

Endotoxin triggers many of the adverse systemic reactions and serious sequelae in patients with septicaemia and septic shock. The clinical presentation is dynamic and variable depending on the severity of the underlying illness, the duration of shock and its aetiology (Easmon, 1990). Clinically, two haemodynamic phases are recognized, although these are not always distinct entities (Dipiro, 1990). The hyperdynamic state, also referred to as early or warm shock, is characterized by elevated temperature and increased respiratory rate and cardiac output. Near normal blood pressure is maintained, despite low systemic vascular resistance. Unsuccessful treatment at this stage is followed by progression to the hypodynamic state, termed late or cold shock. This state is associated with decreasing blood pressure and reduced cardiac output, with decreased tissue perfusion, high systemic vascular resistance and general organ dysfunction. Ultimately, death results from single or multiple organ failure if the process is not reversible (Parrillo *et al*, 1990).

Bone *et al* (1989) proposed the following criteria for defining the

sepsis syndrome: temperature above 38.3°C or below 35.6°C; heart rate above 90 beats per minute; respiratory rate greater than 20 breaths per minute; evidence of infection at any site, and signs of inadequate organ function and perfusion. These criteria are useful in identifying patients before progression to the later stages of septic shock, and were among those used by Ziegler *et al* (1991) for the selection of patients for the clinical trial of an anti-endotoxin antibody.

CHAPTER 2

THE GRAM-NEGATIVE CELL ENVELOPE

The cell envelope of Gram-negative bacteria is morphologically more complex than Gram-positive bacteria (Hammond *et al*, 1984). Maintaining both rigidity and shape on the bacterial cell, it also acts as a barrier through which a bacterium interacts with its environment (Costerton *et al*, 1974; Brown & Williams, 1985). Indeed, the envelope phenotype expressed by a bacterium is a product of its growth environment (Williams, 1988).

The Gram-negative cell envelope (Figure 1) consists of an innermost cytoplasmic membrane, the peptidoglycan layer, the periplasm and the outer membrane. Additional surface structures often present on Gram-negative bacteria include fimbriae, flagella and discrete exopolysaccharide capsules or loosely attached slime layers, (Sutherland, 1977; 1985) sometimes referred to as glycocalyces (Costerton, 1984).

2.1 STRUCTURE AND COMPOSITION OF THE OUTER MEMBRANE

The outer membrane of Gram-negative bacteria represents a unique planar lipid bilayer structure composed of three major components; LPS, protein and phospholipid (Costerton *et al*, 1974; Hammond *et al*, 1984; Nikaido & Vaara, 1985). Phospholipids, occupying the inner face of the bilayer, interact hydrophobically with the lipid component of LPS which forms the majority of the outer face. LPS, representing more than 50% of the weight of extracted outer membrane (Costerton *et al*, 1974), makes an important contribution to the assembly and maintenance of the outer membrane as a permeability barrier while still allowing the

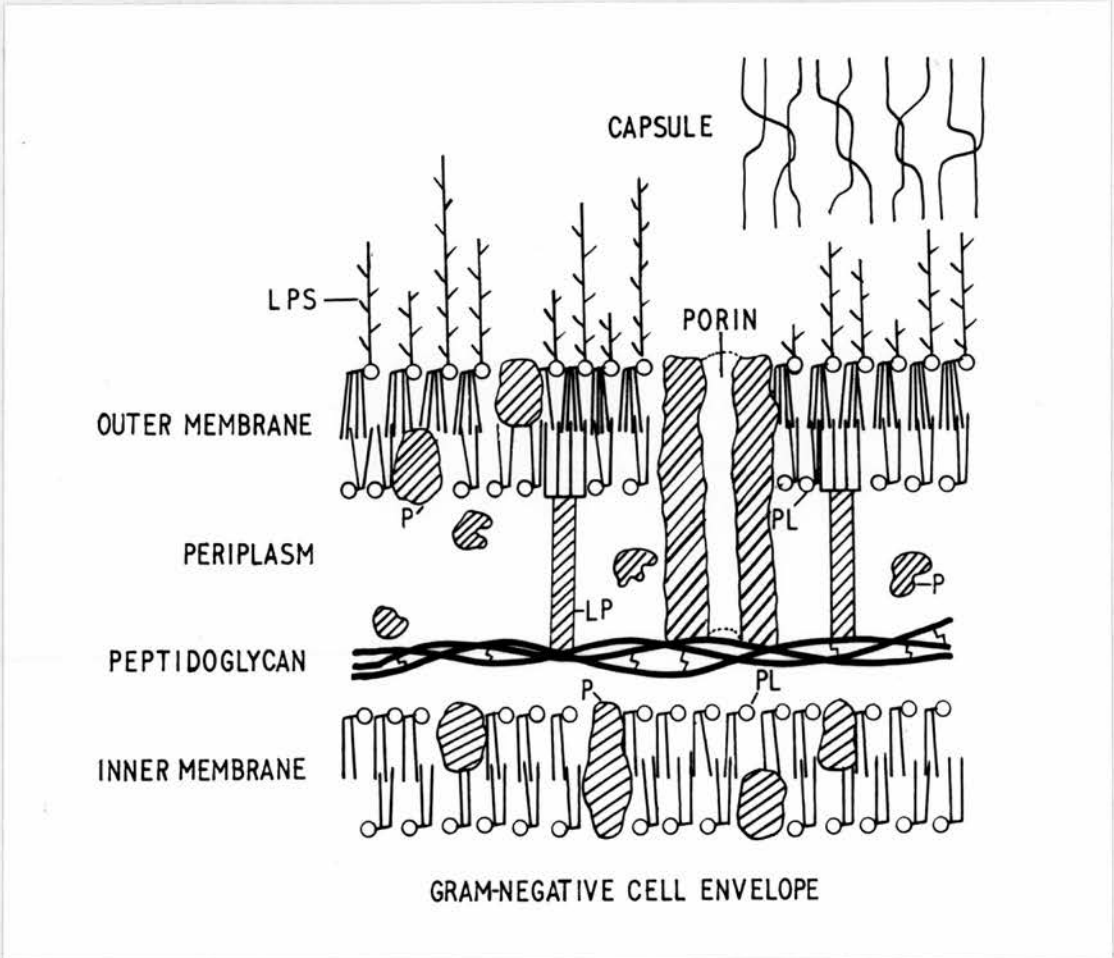


Figure 1. The cell envelope of a Gram-negative bacterium.
LP = lipoprotein; LPS = lipopolysaccharide; P = protein; PL = phospholipid. (From Hancock & Poxton, 1988).

efficient diffusion of nutrients (Hancock, 1984; Nikaido & Vaara, 1985). Zorzopulos *et al* (1989) proposed that LPS is organised in a network of particles which serves as the skeleton of the outer membrane where the net holes are filled with phospholipids and protein. Its central role in the pathogenicity and antigenicity of Gram-negative bacteria is also acknowledged (Wicken & Knox, 1980; Jann & Jann, 1985). The outer membrane also possesses protein components including lipoproteins, which play an important role in maintaining the structure and stability of the outer membrane, and major and minor outer membrane proteins, many of which perform specialized functions (Williams, 1988). Flagella and fimbriae are other proteinaceous appendages exposed at the surface of some Gram-negative bacteria, conferring the functions of motility and attachment respectively.

2.2 LIPOPOLYSACCHARIDE

Lipopolysaccharides are unique, complex amphipathic macromolecules situated in the outer membrane of the cell envelope of Gram-negative bacteria (Mühlrädt & Golecki, 1975; Funahara & Nikaido, 1980). Although sharing the same general composition, LPSs show distinct heterogeneity in size within a bacterium (Munford *et al*, 1980). LPS is composed of three genetically, biochemically and antigenically distinct regions linked covalently together (Figure 2), (Wilkinson 1977; Lüderitz *et al*, 1982). The hydrophobic lipid A region of LPS is a phosphorylated and extensively acylated glucosamine disaccharide embedded in a continuum of proteins and phospholipids of the outer membrane. Extending outwards from lipid A is the common oligo-saccharide core region. In smooth strains, a longer carbohydrate polymer, the O-polysaccharide side chain extends outwards from the core and the cell surface (Westphal *et al*, 1983; Hammond *et al*, 1984). The

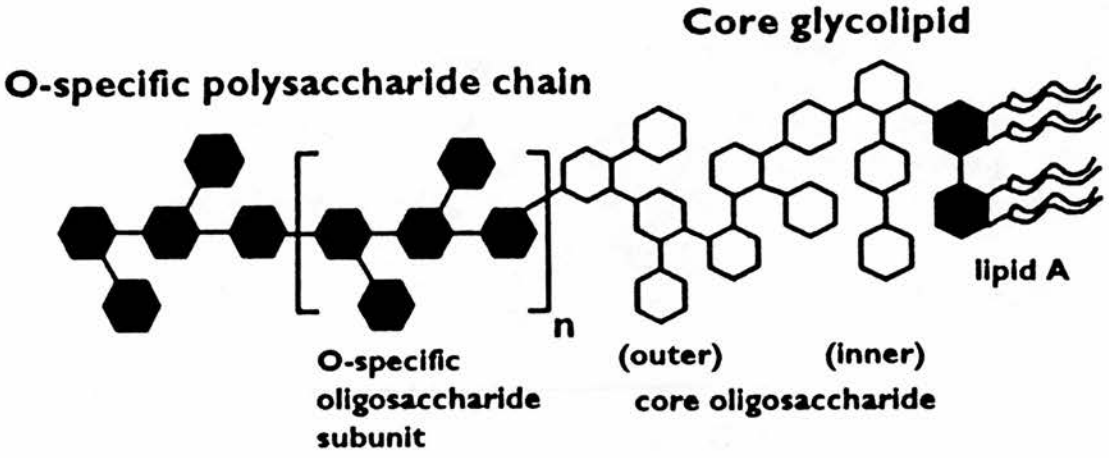


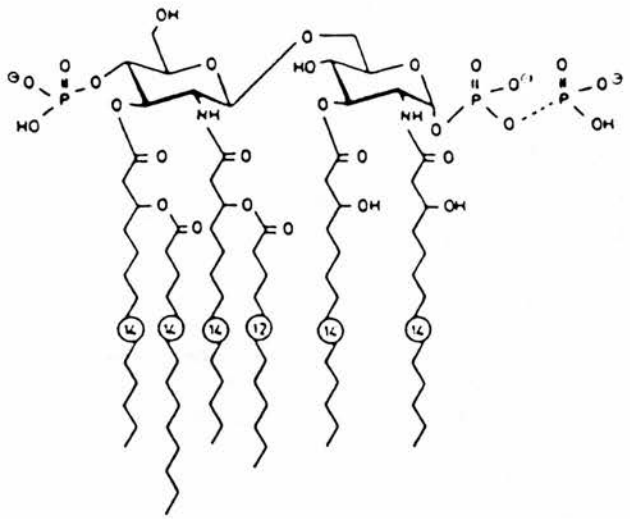
Figure 1. Schematic representation of a smooth lipopolysaccharide, showing the principal structural components.

core and lipid A regions are relatively conserved compared with the extreme structural variability observed among LPS O-side chains of different Gram-negative bacteria. LPS carries a net negative charge resulting in the strong negative surface charge of Gram-negative cells (Sherbert & Lakshmi, 1973).

Lipid A

The lipid A moiety of LPS has the same fundamental structural elements in all Gram-negative bacteria (Brade *et al*, 1988). These consist of a bis-phosphorylated β-1, 6-linked D-glucosamine disaccharide substituted with six or seven saturated fatty acids (Westphal *et al*, 1983; Nikaido & Vaara, 1985). The fatty acids, usually hydrophobic (R)-3-hydroxy-myristic acid, substitute 2, 2′, 3 and 3′ positions of the glucosamine disaccharide in amide and ester linkages respectively (Brade *et al*, 1988). The chemical structures of lipid A from *E. coli* and *Salmonella minnesota* are shown in Figure 3 (Rietschel *et al*, 1984a). In *E. coli* lipid A, the acyl residues of the non-reducing glucosamine at positions 2′ and 3′ are esterified with lauric and myristic acid respectively. These acyloxyacyl residues are typical structural markers of LPS (Brade *et al*, 1988). The genus *Salmonella* contains the same elements as lipid A of *E. coli*, but carries additional substituents (Wollenweber *et al*, 1982; Galanos *et al*, 1986). This lipid A carries phosphoryl-ethanolamine on the C1 phosphate and 4-deoxy-4-amino-L-arabinopyranose on the C4′ phosphate of the hydrophilic backbone, and the amide-linked 3-hydroxymyristic acid of the reducing glucosamine is substituted with palmitic acid, forming a third acyloxyacyl residue. Comparative studies of lipid A from many genera of Enterobacteriaceae have shown that the lipid A structure appears to be closely related but not identical (Mattsby-Baltzer *et al*, 1984). Variations of lipid A

a



b

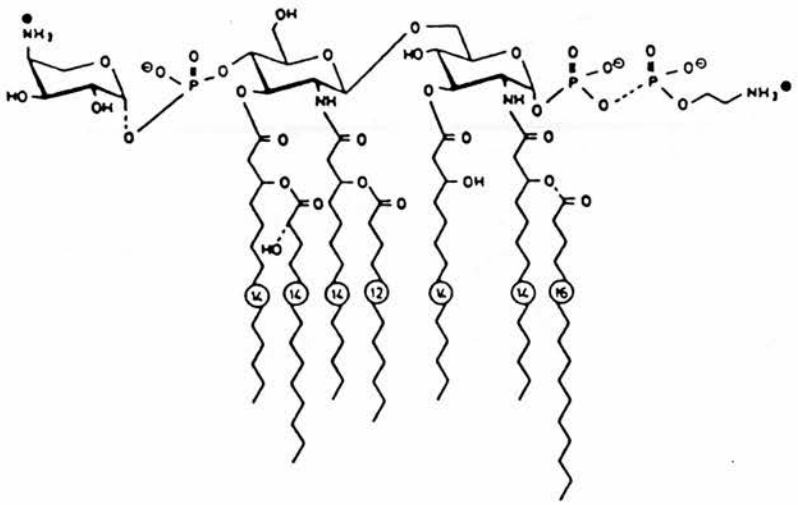


Figure 3. Chemical structure of the *E. coli*-type lipid A (a) and the *Salmonella*-type lipid A (b). The numbers in circles show the number of carbon atoms in acyl chains. (Rietschel *et al*, 1984).

structures concern: the number of carbon atoms in the hydrocarbon chains; the type and distribution of acyloxyacyl residues, and the additional substitution of phosphoryl residues (Brade *et al*, 1988).

The high degree of structural conservation shown between genera makes lipid A the least variable component of LPS (Drewry *et al*, 1973; Westphal *et al*, 1983; Galanos *et al*, 1984). It is now well established that lipid A represents the endotoxic principle of LPS, being responsible for its pathophysiological effects (Galanos *et al*, 1985a; Rietschel *et al*, 1987; Brade *et al*, 1988). The term endotoxin, synonymous with LPS, was introduced in the 19th century to describe the component of Gram-negative bacteria responsible for the patho-physiological phenomena associated with Gram-negative infections (Rietschel *et al*, 1984b; Suffredini *et al*, 1989). A number of structural constituents required for the full expression of endotoxicity of lipid A have been identified (Galanos *et al*, 1984; Homma *et al*, 1985; Matsuura *et al*, 1985; Brade *et al*, 1987a). Small changes in the structure of lipid A (native or synthetic) can lead to a marked reduction in toxicity (Proctor *et al*, 1986; Kiener *et al*, 1988).

Core oligosaccharides

The core region consists of a hetero-oligosaccharide divided into inner and outer subdomains. Structural analysis of the core oligosaccharides has been achieved through studies of a number of rough mutant strains from various organisms (Ørskov *et al*, 1977; Westphal *et al*, 1983; Brade *et al*, 1988). Rough (R)-form variants, so called because of their colony morphology, represent bacteria lacking the O-side chain and possibly parts of the core oligosaccharide as a result of biosynthetic defects. Based on the sugar composition of the LPS isolated from

various sub-mutants of R-form bacteria with incomplete core oligosaccharides it is possible to construct a classification of LPS submutants, termed chemotypes.

The chemotypes for R-submutant LPS of the *Salmonella* core-glycolipid (CGL) are shown in Figure 4. The Ra mutant represents the full core oligosaccharide with each subsequent mutant possessing progressively shorter oligosaccharides substituted onto lipid A. The Re chemotype is the smallest naturally occurring LPS mutant, possessing only the lipid A and 3-deoxy-D-manno-2-octulosonate (KDO).

The inner core exhibits a high degree of structural conservation among all Enterobacteriaciae (Eskenazy *et al*, 1977; Jansson *et al*, 1981; Perez-Perez *et al*, 1986). It is composed of two unusual sugars, heptose (mainly in the L-glycero-D-manno- and the D-glycero-D-manno-configuration) and KDO which links the core polysaccharide to lipid A via an acid labile bond (Wicken & Knox, 1980). In addition, charged residues such as phosphate and ethanolamine are common constituents of the inner core (Brade *et al*, 1988).

The KDO region of the LPS appears to be indispensable, since mutants defective in the biosynthesis of the inner core region have never been isolated (Hammond *et al*, 1984). The structural elucidation of the KDO region could not be approached for a long time due to difficulties with the chemistry of KDO (Brade *et al*, 1988), leading to controversy in its structure. Lüderitz *et al* (1966) proposed one KDO residue for the main sugar chain, whilst Prehm *et al* (1975) later reported two KDO residues in the main chain. The substitution of the inner KDO residue (next to lipid A) by a third KDO residue was later described by Jansson *et al*

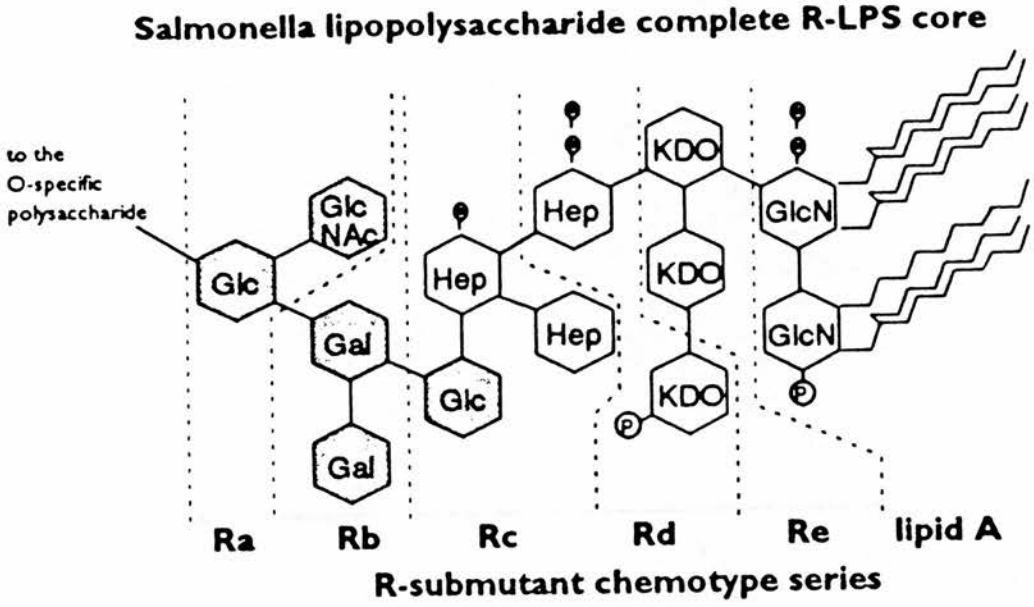


Figure 4. Rough Lipopolysaccharide Chemotypes of *Salmonella*.

(1981). Finally, it is now thought that Re mutants (lipid A and KDO alone) contain two KDO molecules in the α pyranoid ring form and 2-4 interlinked, hence only one KDO residue in the main sugar chain. In mutants other than Re, the reducing KDO (KDO I) is substituted at position 5 by a heptose and in position 4 of the terminal KDO by a third KDO in α pyranosidic linkage (KDO III), (Brade & Rietschel, 1984; Brade *et al*, 1985; 1986; 1987b; 1988). However, substitution with the third KDO is often not present in stoichiometric amounts (Brade *et al*, 1988). Indeed, although the third KDO, the third heptose and several phosphorylated substituents are thought to exist, the techniques used to show their presence are indirect and subject to analytical artefacts (Tacken *et al*, 1986; Raetz, 1990).

The outer core contains the frequently occurring hexoses, D-glucose, D-galactose, and N-acetyl-D-glucosamine in different proportions and sequential arrangements, accounting for the main differences between the known core types (Figure 5) (Schmidt *et al*, 1970; Jansson *et al*, 1981; Lüderitz *et al*, 1982; Westphal *et al*, 1983; Perez-Perez *et al*, 1986; Barclay, 1990). The linkage of the outer core to the O-side chain is another variable between LPS (Jansson *et al*, 1981). Although most of the structural variations in the core occurs in the outer region, these are low compared to that of the O-side chain. Thus, in *Salmonella* species only one core type exists and in *E. coli* only five core types have been described (Hammerling *et al*, 1971; Prehm *et al*, 1976; Feige *et al*, 1977; Jansson *et al*, 1981; Westphal *et al*; 1983; Brade *et al*, 1988).

The core of *P. aeruginosa* differs significantly from other described LPSs (Day & Marceau-Day, 1982). In contrast to entero- bacterial LPS

Core type	Structure
Salmonella	αD-Glc*p*NAc(1 → 2)αD-Glc*p*(1 → 2)αD-Gal*p*(1 → 3)αD-Glc*p*(1 → 6 ↑ 1 αD-Gal*p*
E. coli R1	αD-Gal*p*-(1 → 2)αD-Gal*p*(1 → 2)αD-Glc*p*(1 → 3)αD-Glc*p*(1 → 3 ↑ 1 βD-Glc*p*
E. coli R2	αD-Glc*p*NAc(1 · · · > 2)αD-Glc*p*(1 → 2)αD-Glc*p*(1 → 3)αD-Glc*p*(1 → 6 ↑ 1 αD-Gal*p*
E. coli R3	αD-Glc*p*(1 → 2)αD-Glc*p*(1 → 2)αD-Gal*p*(1 → 3)αD-Glc*p*(1 → 3 ↑ 1 αD-Glc*p*NAc
E. coli R4	αD-Gal*p*(1 → 2)αD-Gal*p*(1 → 2)αD-Glc*p*(1 → 3)αD-Glc*p*(1 → 4 ↑ 1 βD-Gal*p*
E. coli K12	βD-Glc*p*NAc(1 · · · > 6)αD-Glc*p*(1 → 2)αD-Glc*p*(1 → 3)αD-Glc*p*(1 → 6 ↑ 1 αD-Gal*p*

Figure 5. Lipopolysaccharide core oligosaccharides.

which contains about four phosphate residues in the inner core region and an additional two or three in the lipid A, LPSs from *P. aeruginosa* may contain ten or more residues per molecule (Kropinski *et al*, 1979; Wilkinson, 1983; Kropinski *et al*, 1985). The outer core of *P. aeruginosa* typically contains D-glucose and D-galactosamine with the addition of L-rhamnose and alanine (Wilkinson, 1983).

It has been proposed that rough mutant bacteria or their LPS induce specific core or R antibodies since, as for O-polysaccharides, the terminal sugar residues represent the immunodominant sugar (Brade *et al*, 1988). However, in the host, natural exposure will only occur to the complete unsubstituted core LPS (Ra chemotype) and core LPS substituted with O-polysaccharide (smooth bacteria) (Barclay, 1990).

O-side chains

The O-side chain component of LPS is a polysaccharide chain consisting of repeating oligosaccharide subunits which extend outwards from the cell surface in an irregular spiral shape. The side chains of most enteric bacteria consist of a variable number of repeating tri-pentasaccharides which can range from one to as many as forty units (Williams, 1988). Such heterogeneity in the chain length of the O-specific polysaccharide can be demonstrated with the aid of sodium-dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). This allows LPS molecules differing by one O-antigen repeat to be separated from each other (Goldman & Leive, 1980; Palva & Mäkelä, 1980) permitting estimation of the extent of O-antigen polymerization.

The number of repeating units can show considerable variation under different physiological conditions (Chester & Meadow, 1975; McGroarty

& Rivera, 1990). Such variation provides the bacterium with a means to modify its surface properties and thereby its interaction with various agents in the environment (Nikaido & Nakae, 1979; Munford *et al*, 1980; Taylor, 1983).

Most repeating units are hetero-oligosaccharides, usually containing short chain branches, although homo-oligosaccharides in the linear chain formation have been shown to exist, including *E. coli* O8 and O9 (identical with those of *Klebsiella* O5 and O3 respectively) containing 2- and 3- linked mannose residues (Westphal *et al*, 1983; Jann & Jann, 1987). *Salmonella typhimurium* possesses a tetrasaccharide repeating unit containing the 2-deoxy-hexoses (rhamnose & abequose) and the hexoses galactose and mannose (Hammond *et al*, 1984). The O-side chains of many other salmonellae also contain galactose, mannose and rhamnose often in an identical sequence, although their linkage may be either the α or β configuration. In contrast to *Salmonella* and *Klebsiella*, which produce only neutral O-specific polysaccharides, *E. coli* can also express acidic O-specific polysaccharides (Jann & Jann, 1987). In most LPS the acidic constituents are hexuronic acids, whilst the specific neutral polysaccharide of *E. coli* often contain unusual amino sugars and rhamnose.

Variation in both the composition and combinations of sugars in the repeat unit, together with differences in the position and anomeric configuration of their glycosidic linkages, creates immense structural diversity per unit structure of the O-side chain (Barclay, 1990). Such variability within this component of LPS is reflected in the ability to classify strains of Gram-negative organisms by their O-antigen, resulting in a number of distinct O-serotypes. There are over 160

serotypes in *E. coli*, over 60 types in *Salmonella*. Liu *et al* (1983) proposed a scheme comprising 17 O-types for *P. aeruginosa*, although three new major somatic antigens have recently been described (Liu & Wang, 1990). The many modifications of O-antigens may help Gram-negative bacteria to evade the immune system (Raetz, 1990) and their presence has also been associated with resistance to serum complement and virulence in many species of Gram-negative bacteria including *E. coli, Salmonella, Shigella* (Taylor, 1983; Cross *et al*, 1986) and *Klebsiella* (Williams *et al*, 1983; Tomas *et al*, 1986).

It has been demonstrated that the O-antigen of different *E. coli* serotypes show serological cross-reactivity indicating recognition of similar epitopes in the different O-polysaccharide antigens (Ørskov *et al*, 1977; Pluschke *et al*, 1986). The commonest serogroups of *E. coli* in septicaemic patients include O1, O2, O4, O6, O15, O18 (Eykyn *et al*, 1990; Gransden *et al*, 1990). *E. coli* O18, frequently associated with neonatal infections contains several serologically related but not identical O-antigens, including O18 ab and O18 ac. The most abundant O18 antigen of invasive *E. coli* is the O18 ac antigen (Gupta *et al*, 1984).

Compared to *P. aeruginosa* the overall capping frequency of enterobacterial LPS (ie the percentage of core LPS constituents covered with O-antigen) is high, leaving a lower percentage of core determinants exposed at the cell surface (Kropinski *et al*, 1985). The percentage of smooth type molecules in *P. aeruginosa* LPS has been reported at 8% (Rivera *et al*, 1988) compared to 35% and 65% in *S. typhimurium* and *E. coli* respectively (Goldman & Leive, 1980; Palva & Mäkelä, 1980). The LPS of other pathogenic bacteria, such as *Neisseria gonorrhoeae* and

Haemophilus influenzae, which do not contain any O-antigen is often termed lipooligosaccharide (LOS).

2.3 BIOSYNTHESIS OF LIPOPOLYSACCHARIDE

Advances in the determination of the mechanisms involved in the synthesis of LPS have been greatly facilitated by the isolation of the biosynthetic mutants blocked at various points of the assembly sequence, and by the use of specific inhibitors (Hammond *et al*, 1984). The mechanisms involved in the synthesis of the lipid A, core and O-side chain have been determined at both biochemical and genetic levels (Osborn *et al*, 1972; Ørskov *et al*, 1977; Ishiguro *et al*, 1986; Brahmbhatt *et al*, 1988).

Since very few mutants defective in lipid A have been described, knowledge of the assembly sequence of the lipid A-KDO region is incomplete. An important precursor in the biosynthesis of lipid A is a diacylated glucosamine-1-phosphate, termed lipid X which accumulates to high levels in some phosphatidylglycerol-deficient mutants of *E. coli* (Nishijima *et al*, 1981; Nishijima & Raetz, 1981). Ray *et al* (1984) demonstrated that by incubating 1 mM lipid X and 1 mM UDP-2, 3-diacyl-GlcN with a crude cytosolic fraction of *E. coli* a new product was formed with the characteristic β, 1-6 linkage of lipid A. The identification of the high energy donor UDP-2, 3-diacyl-GlcN as a natural product in living cells supported the evidence that lipid X was a precursor of lipid A. KDO on the other hand is activated to CMP-KDO, a sugar nucleotide that serves as the precursor of the KDO domain of LPS. Incorporation of KDO into the lipid A precursor precedes the incorporation of ester-linked straight chain fatty acids (Rick *et al*, 1977; Rick & Osborn, 1977). Although a full understanding of the

biosynthetic pathway of lipid A is still incomplete, further information is being generated as the structural requirement for toxicity and immunogenicity become clearer (Anderson *et al*, 1985; Coleman & Raetz, 1988; Brozek *et al*, 1989; Galloway & Raetz, 1990).

The R-core is sequentially assembled at the cell membrane by the transfer of sugars, phosphate and ethanolamine to the lipid A-KDO moiety. The mechanism by which the heptose units are added to the fully acylated lipid A-KDO is obscure (Hammond *et al*, 1984). The hexoses of the outer core are added, one residue at a time from UDP derivatives which function as activated sugar donors (Osborn, 1969; Rothfield & Romeo, 1971). The glycosyl transferases that generate the outer core are peripheral membrane proteins requiring phosphatidylethanolamine for activity (Osborn, 1969; Rothfield & Romeo, 1971; Hinckley *et al*, 1972; Raetz, 1990).

The O-side chain is synthesized independently of lipid A and core by a series of cytoplasmic membrane bound enzymes (Jann & Jann, 1984). Its repeating oligosaccharide unit is synthesized on a 55-carbon isoprenoid carrier lipid (Osborn, 1969; Rothfield & Romeo, 1971). An important feature of O-antigen polymerization is that the newly generated tetrasaccharide repeat is incorporated at the reducing end of the growing chain (Raetz, 1990). Finally, an LPS ligase transfers O-antigen to the outer core independent of the length of the O-side chain.

The mechanisms involved in the translocation and subsequent introduction of a molecule as complex LPS into the outer membrane is still open to debate, although is thought to occur through adhesions between the

two membranes (Hammond *et al*, 1984; Raetz, 1990).

2.4 PROTEINS OF THE OUTER MEMBRANE

Of the three major classes of protein found in the outer membrane, lipoproteins are the most abundant, accounting for approximately six per cent of total cell protein (Hammond *et al*, 1984). Lipoproteins play an important role in anchoring the outer membrane to the cell wall, and in maintaining the structure and stability of the outer membrane (Brass, 1986).

The term major outer membrane protein, refers to components present in sufficient quantity to be readily detected by simple staining of polyacrylamide gels. These include the pore proteins termed porins (in *E. coli* K12, Omp F and Omp C; in *S. typhimurium* LT2, Omp F, Omp C, and Omp D proteins) and Omp A proteins. The porins form relatively non-specific, water-filled pores spanning the outer membrane for the passive entry by low molecular weight hydrophilic solutes present at relatively high concentrations (around 10^{-6} M) (Nikaido & Nakae, 1979; Nikaido & Vaara, 1985; Nakae, 1986). The porins also serve as receptors for bacteriocins and bacteriophages (Lugtenberg & Van Alphen, 1983). The Omp A protein is associated with lipoprotein and plays a role in conjugation and the stability of the outer membrane (Vaara & Nikaido, 1984).

Some of the minor outer membrane proteins are constitutive but many are inducible or derepressible and may, under appropriate environmental conditions, reach levels with those of the major proteins. Many of the minor proteins facilitate specific diffusion processes of solutes which are too large to pass through the porins and which are present in low

concentrations (down to 10^{-2} M). The process involves the binding of substrate or a substrate chelator complex to these outer membrane receptor proteins (Brass, 1986; Williams, 1988). An important example of this is the uptake of ferric ions chelated to low molecular weight iron chelating compounds (siderophores) via specific inducible outer membrane receptor proteins (Neilands, 1981; 1982). This process plays an integral role towards a bacterium's ability to multiply in host tissues during infection.

2.5 EXOPOLYSACCHARIDE

Gram-negative bacteria growing in the natural environment or pathogens causing bacteraemia, meningitis and urinary tract infections are usually extensively covered by discrete exopolysaccharide capsules or loosely attached slime layers (Williams, 1988). Capsules have evolved distinctive structural and functional characteristics which are of cardinal importance in the pathogenesis of infections such as septicaemia. These include the protection of most organisms against the non-specific host defence which is exerted in the preimmune phase of infection by serum complement and phagocytes as described in Section 4.2.

Capsules are glycoconjugates composed primarily of monosaccharides forming linear homopolymers of a single sugar, linear copolymers composed of two or more monosaccharides, or multi-chained branched polymers composed of at least five monosaccharides (Robbins, 1978; Jennings, 1983; Silver & Vimr, 1990). Many capsules are acidic due to the possession of carboxyl groups, either from acidic sugars such as uronic acids or neuramic acid, or from non-sugar substituents such as pyruvyl, acetyl and formyl groups (Poxton & Arbuthnott, 1990).

The typing of capsules is largely based upon serological analysis of the capsular antigen which has demonstrated the wide variety of antigenic types produced by different strains of the same species (Hammond *et al*, 1984). Serological studies on *E. coli* (Kauffmann & Vahlne, 1945; Ørskov *et al*, 1977) demonstrated additional surface antigens covering the cell wall O-antigens which were assumed to be present in an extracellular envelope or capsule. They were termed K antigens ("Kapselantigene"). A limited number of acidic K antigens predominate among invasive *E. coli* (Ørskov *et al*, 1977). K1, K2, K3, K5, K12 and K13 antigens accounting for the majority of all *E. coli* isolates from the upper urinary tract (Kaijser *et al*, 1977) and K1 comprising almost all of isolates from neonatal septicaemia (Schiffer *et al*, 1976).

The capsular antigens of *E. coli* are acidic polysaccharides which can be divided into two groups on the basis of chemical, physical and microbiological characteristics. The high molecular weight polysaccharides of group I have a relatively low charge density/low electrophoretic mobility, contain hexuronic acids as the acidic component, and are co-expressed essentially with only the O8 and O9 antigens at all growth temperatures (Jann & Jann, 1987). Structural and serological studies have revealed that these polysaccharides may occur on the *E. coli* surface as either a group I capsular polysaccharide or as an acidic LPS, forming the cell wall O-antigen (Ørskov *et al*, 1977; Jann & Jann, 1983; 1984). Capsules of *Klebsiella* share many properties with the polysaccharides of this group, indicating an inter-generic relationship similar to that described with the respective O-antigens (Jann & Jann, 1990).

Whilst group I capsular polysaccharides are a relatively homogeneous group, the capsular polysaccharides of group II, which are present on most of the strains causing extraintestinal infections, show great variations in composition and general structural features. The majority are heteropolysaccharides containing more unusual acidic compounds such as KDO, N-acetylneuraminic acid (NeuNAc), N-acetylmannosaminuronic acid (ManNAcUA) or phosphate (Jann & Jann, 1987). Of the several capsular polysaccharides containing Neu NAc, two are homopolysaccharides, differing only in linkage position. These Neu NAc polymers, termed sialic acids, occur as the capsule of *E. coli* K1 (Dewitt & Rowe, 1961) and *Neisseria meningitidis* b (Kasper *et al*, 1973), both highly virulent organisms. The virulence of K1 *E. coli* is thought to be based on the ability of the K1 capsule to mask underlying structures on the bacterial cell that activate the antibody independent alternative complement pathway (Horwitz & Silverstein, 1980), and its poor immunogenicity (Cross, 1990) (see Section 4.2).

Infections due to K1 capsulate *E. coli* produce significant morbidity and mortality especially in neonates (McCracken & Mize, 1976; Cross *et al*, 1984). The K1 capsule associates with only a relatively few serotypes. Four O-antigens (O7, O16, O18 and O83) account for the majority of K1 strains isolated from infected neonates (Cheasty *et al*, 1977; Korhonen *et al*, 1985), whilst Cross *et al*, (1984) established that a large proportion of K1 positive *E. coli* isolated from blood and cerebral spinal fluid were associated with the O-antigens of O1, O2, O12, O16 and O18ac. This association of specific capsules with only a limited number of O-serogroups is believed to be evidence of the existence of bacterial clones of common origin (Ørskov *et al*, 1976; 1990). It has been proposed that many of these clones survived because

of unique functional relationships between capsules, LPS and possibly other determinants in that clone, allowing the bacterial clone to compete and survive (Cross, 1990). The K1 antigen has been found to undergo phase variation of O-acetylation of sialosyl residues, covalent modifications which may undergo transition between acetylated and unacetylated states (Ørskov *et al*, 1979).

The mode of attachment of capsule to the cell surface is still largely unanswered. However, the relatively recent finding of a lipid on the reducing end of some *E. coli* capsular polysaccharide was thought to suggest that they associate with the outer membrane through hydrophobic interactions. It is thought that group I polysaccharides are bound to core-lipid A, whilst the lipid end of group II capsular polysaccharides was identified as a phosphatidic acid bound in a very labile linkage (Jann & Jann 1987; 1990). In all group II polysaccharides which contain KDO in their repeating unit, KDO is the reducing sugar (Schmidt & Jann, 1982) whilst NeuNAc is the reducing end for the polysialyl antigens of, for example, *E. coli* K1 (Gotschlich *et al*, 1981). Since not all group II polysaccharides are lipid substituted Jann & Jann (1990) proposed that the lipid-substituted polysaccharide fraction is anchored in the bacterial outer membrane by hydrophobic interactions, and the unsubstituted polysaccharide is retained by ionic and other interactions.

2.6 FIMBRIAE

Fimbriae (pili) of Gram-negative bacteria are broadly classed as mannose sensitive (MS), or mannose resistant (MR), depending upon whether or not the adhesion is inhibited by D-mannose. Fimbriation is a reversible trait most likely determined by chromosomal genes and

present up to 1,000 per bacterium (Hammond *et al*, 1984).

Fimbriae may assist in the attachment of organisms to and invasion of various cell surfaces, an essential part of the establishment of many bacterial infections. Work by Saukonen *et al* (1988) determined that fimbriae aided the adherence of cells to vascular endothelium during invasive infections, particularly septicaemia, therefore preventing the removal of bacteria by phagocytosis. This could progress to local tissue damage, and the release of mediators from the endothelial tissue and the cellular immune system as part of the septic syndrome (Steadman *et al*, 1988).

2.7 FLAGELLA

Flagella are protein, filamentous cell surface appendages responsible for the mobility and chemotaxis of many bacteria (Doetsch & Sjoblad, 1980). Despite having key roles to play in some invasive infections, their importance to the pathogenesis of septicaemia is thought to be insignificant.

CHAPTER 3

ENVIRONMENTAL MODULATION OF THE GRAM-NEGATIVE CELL ENVELOPE

3.1 PLASTICITY OF THE BACTERIAL CELL ENVELOPE

The ability of an invading bacterium to adapt rapidly to the host environment is widely recognized as a key determinant of virulence and pathogenicity (Smith, 1990). The cell envelope of pathogenic bacteria plays a pivotal role in this adaptive process, since it is involved in promoting adhesion and colonization of host tissues, in the acquisition of essential nutrients and in conferring resistance to host defences and to antibiotics (Brown & Williams, 1985). Many environmental variables, in particular, nutrient deprivation, growth rate, growth temperature, and replication within a surface associated biofilm all exert a marked effect *in vivo* upon the macromolecular structure and function of the cell envelope (Williams, 1988). It is this remarkable plasticity of the bacterial cell envelope which confers a distinct survival advantage to the organism as it moves from one environmental niche to another within the host.

Cell envelope properties are ultimately determined by information contained within the genome which also has the capacity to respond to environmental changes (Harder & Dijkhuizen, 1983). This may occur in principle by changes in genetic constitution, phase variation or phenotypic adaptation. However, bacteria only express that part of their genome enabling them to adapt functionally and structurally to their immediate environment, and a wide variety of phenotypic responses may be encountered within a specific genotype (Harder & Dijkhuizen, 1983; Williams, 1988; Miller *et al*, 1989). Phenotypic adaptation is

characterized by a change in expression of one or more genes in response to an environmental stimulus, without any alterations to the DNA coding sequence (Clark, 1990). Although there are many examples of environmental regulation of various gene products, it is only relatively recently that attention has focused on the identification and characterization of the regulatory mechanisms associated with the expression of virulence factors in the host environment (Finlay & Falkow, 1989). Some pathogens, have been demonstrated to regulate the production of various virulence determinants by common mechanisms which are influenced by environmental factors (Betley *et al*, 1986; Miller *et al*, 1989). The induction or repression of diverse and unlinked genes are known to be co-ordinated by environmentally controlled global regulatory networks (Gottesman, 1984), and appears to involve DNA supercoiling and protein phosphorylation (Dorman *et al*, 1988; Bhriain *et al*, 1989; Bourret *et al*, 1989).

The knowledge of the molecular mechanisms governing global regulation of pathogenicity is integral to the understanding of bacterial infectious processes (Miller *et al*, 1989). The virulence determinants of *Bordetella pertussis* are all regulated by a single genetic locus, vir, which is negatively regulated by temperatures of <37°C and increased levels of nicotinic acid and $MgSO_4$ (Weiss *et al*, 1983; Weiss & Hewlett, 1986). Products of this locus function as a positive inducer of many virulence genes, including those which encode filamentous haemagglutin , pertussis toxin, adenylate cyclase, haemolysin, fimbria subunits, and dermonecrotic factors. This regulatory mechanism is related to several two-component bacterial regulatory systems which transcriptionally regulate several genes (Finlay & Falkow, 1989). These systems similarly respond to environ-

mental stimuli and control, for example, chemotaxis, phosphate response, and osmotic response in *E. coli* and sporulation in *Bacillus subtilis* (Nixon *et al*, 1986; Ronson *et al*, 1987; Miller *et al*, 1989).

3.2 THE *IN VIVO* ENVIRONMENT - NUTRIENT DEPRIVATION

The ability of microorganisms to adjust both structurally and functionally to changes in their environment is particularly applicable to their behaviour in response to the most common of environmental constraints, that of nutrient deprivation (Harder & Dijkhuizen, 1983). Essential nutrients or environmental factors which are most critical in influencing particular pathogens *in vivo* are relatively unknown, and unstudied (Smith, 1990).

The most notable exception to the lack of knowledge is the comprehensive work on iron limitation. Iron is a crucial nutrient for most bacteria, and its availability is severely restricted within the host (Brown & Williams, 1985; Griffiths, 1987). The form and abundance of iron compounds varies significantly within the mammalian host, but free iron is rarely available (Clark, 1990). Most iron is stored intracellularly, predominantly as haem, but also as a component of non-haem proteins, or stored as ferritin. Extracellulary, iron is generally complexed to high-affinity iron-binding glycoproteins, transferrin and lactoferrin, which have association constants for iron of about 10^{36} (Aisen & Leibman, 1972). Iron availability is further limited during infection by inhibitory iron absorption in the gastrointestinal tract, a phenomenon known as "hypoferraemia" (Weinberg, 1984). This state is mediated by interleukin-1 (IL-1), induced by the exposure to endotoxin (Kluger & Bullen, 1987). The early release of lactoferrin by leukocytes during degranulation and

phagocytosis during infection also reduces iron availability (Leffell & Spitznagel, 1975; Wright & Gallin, 1979) and may be mediated indirectly by IL-1 and tumour necrosis factor (TNF) (Goldblum *et al*, 1987; Kluger & Bullen, 1987; Koivuranta-Vara *et al*, 1987). Lactoferrin can remove iron from transferrin and the iron-bound lactoferrin is rapidly cleared by the reticuloendothelial system. Indeed, the rapid fall of total iron seen in *E. coli* endotoxaemic and septicaemic pigs was explained by this release of lactoferrin and the removal of iron-bound lactoferrin (Gutteberg *et al*, 1989).

The limited availability of iron within serum was illustrated early on by Schade & Caroline (1946). They showed that serum inhibited the growth of *Shigella dysenteriae*, and the inhibition was specifically reversed by the addition of iron. In experimental animals, iron restriction can also be overcome by injection of iron compounds (Bullen, 1981). The ability of the host to respond to infection by restricting the availability of an essential nutrient, has been termed "nutritional immunity" (Kochan, 1977).

Lack of nutrients other than iron which are thought to influence the pathogen include carbon (Smith, 1977), magnesium (Broughton *et al*, 1968; Davey *et al*, 1985), phosphate (Vasil *et al*, 1985) and zinc (Weinberg, 1978). Materials probably not limiting *in vivo* may equally affect the virulence determinants of pathogens. Indeed, recognition that certain nutrients may not be growth limiting *in vivo* on occasion, may direct more attention to non-limiting substrates (Smith, 1990). A given set of growth conditions are also not necessarily universal within a host.

3.3 ENVIRONMENTAL EFFECTS ON THE EXPRESSION OF VIRULENCE FACTORS

The complex and constantly changing *in vivo* environment of bacterial pathogens influences bacterial pathogenicity by affecting growth and the production of virulence determinants (Smith, 1990). Lack of essential nutrients for bacteria results in marked changes in biochemistry and in envelope structure, characteristic of the specific nutrient and with corresponding changes in envelope-associated properties (Brown & Williams, 1985). Growth rate per-se also significantly influences envelope structure and function, with evidence indicating that doubling times *in vivo* are usually relatively long. However, slow multiplication does not necessarily mean that limiting nutritional conditions exist (Smith, 1990). Host defences may restrict growth rate as well as remove or destroy the pathogen.

Pathogenic bacteria overcome iron-restriction by expressing high affinity iron-uptake systems, usually based on low molecular weight iron-chelating agents (Williams, 1988). These siderophores are capable of removing iron from transferrin and lactoferrin, and transporting it to the bacterium via specific iron-regulated OM receptor proteins (Neilands, 1981; 1982) (Section 2.4). Several new OM receptor proteins are synthesized by Gram-negative bacteria under iron-restricted conditions. *E. coli* recovered directly from the peritoneum of lethally infected pigs (Griffiths, 1983), and a mucoid *P. aeruginosa* strain from the lungs of a cystic fibrosis (CF) patient (Brown *et al*, 1984) have both expressed these high affinity iron uptake systems. Other nutrient limitation specific proteins have been obtained for the OM of *K. pneumoniae* during potassium, carbon, phosphate and sulphate restricted conditions (Robinson & Tempest, 1973; Sterkenburg *et al*, 1984; Lodge *et al*, 1986).

LPS structure can be altered phenotypically in response to different environmental factors. Growth rate and nutrient deprivation have been reported to influence the structure of LPS in *E. coli* (Dodds *et al*, 1987). *P. aeruginosa* from CF patients frequently possess non typable, R-LPS (Hancock *et al*, 1983), which lacks the normal O-side chain that protects Gram-negative bacteria from the bactericidal effect of human serum. These observations suggest that in chronic bronchopulmonary infection with *P. aeruginosa* in CF, the organism undergoes an environmental adaptation, marked by changes in somatic antigens and serum sensitivity (Penketh *et al*, 1983). McGroarty & Rivera (1990) demonstrated the alteration of size heterogeneity of *P. aeruginosa* PAO1 serotype LPS under near growth limiting conditions, allowing exposure of common antigen LPS. The ability of an opportunistic pathogen like *P. aeruginosa* to modify the LPS structure, perhaps under the influence of environmental factors (Day & Marceau-Day, 1982), may give the organism a survival advantage and help it persist within the lung.

Exopolysaccharide production is also influenced by nutrient limitation, growth rate and growth temperature (Ørskov *et al*, 1984; Nikaido & Vaara, 1985; van Verseveld *et al*, 1985). The possibility that environmental factors within CF bronchial secretions might induce or switch on the production of alginate and hence influence the emergence of mucoid *P. aeruginosa* has been proposed. Contributory factors include the presence of dipalmitoyl-phosphatidylcholine, the major lung surfactant (Govan, 1975); increased osmolarity (Berry *et al*, 1988; Govan, 1990); effects of individual cations (Boyce & Miller, 1982); nutrient limitation (Speert *et al*, 1990; Terry *et al*, 1991); selection of phage (Govan, 1975) and antimicrobial pressure (Govan & Fyfe, 1978; Deretic *et al*, 1986).

Phenotypically induced alterations in the microbial cell surface affect the susceptibility of the bacterium to antimicrobial agents. In *P. aeruginosa*, magnesium deficiency resulted in the induction of OM protein H1, and associated with increased resistance to polymyxins, aminoglycosides, and ethylenediaminetetraacetic acid (EDTA) (Nicas & Hancock, 1980). H1 has been suggested to replace magnesium at LPS divalent cation cross-bridging sites in the OM, the proposed target sites for these antimicrobials (Nicas & Hancock, 1980; Hancock, 1984). The rate of antibiotic-induced killing has also been shown to decrease in strict proportion to the decrease in the rate of bacterial growth (Tuomanen *et al*, 1986). The mechanism(s) involved, though poorly understood, probably include such contributory factors as OM permeability changes; total cell concentrations or affinities of penicillin-binding proteins, and cell wall synthesizing enzymes.

In addition to direct inhibitory effects, antibiotics at sub-minimum inhibitory concentrations (sub-MICs) can significantly influence the sensitivity of a pathogen to host defences, sometimes as a result of influencing envelope structure (Williams, 1988; Darveau & Cunningham, 1990). The formation or expression of O-side chains and the acidic capsular polysaccharide of *E. coli* is affected by growth of the organism in mecillinam (Taylor *et al*, 1982). The reduction in the envelope polysaccharide components was thought to play a role in the conversion of serum-resistant *E. coli* to serum susceptibility. Similarly, growth of *K. pneumoniae* in the presence of cephalosporins reduced capsule production (Kadurugamuwa *et al*, 1985b) an effect which may increase the susceptibility of an organism to phagocytosis. Antibiotic induced alterations to LPS and other surface structures also frequently correlate with an increase in microbial resistance to

antibiotic therapy (Legakis *et al*, 1989; Pagani *et al* 1990).

Since the genetic basis for virulence is only expressed completely during growth *in vivo*, bacteria grown in most *in vitro* conditions are likely to be incomplete in terms of the full expression of their virulence determinants (Williams, 1988). Thus, a greater understanding of the pathogenesis of disease requires knowledge of the environment at the site of infection, allowing the use of *in vitro* growth conditions that closely mimic the *in vivo* conditions (Clark, 1990).

CHAPTER 4

PATHOGENESIS OF SEPTICAEMIA

Pathogenicity has been defined as the ability of microorganisms to produce disease in susceptible hosts (Smith, 1977). A microbial pathogen is now recognized as a highly adapted microorganism that may cause disease, since its survival depends on a requirement for infection (Falkow, 1990). Since disease is an inadvertent and unfavourable outcome of a microbial infection, the usual outcome is sufficient multiplication by a pathogen to secure its establishment within the host or to bring about its successful transmission to a new susceptible host (Finlay & Falkow, 1989; Falkow, 1990). Not all pathogens however, have an equal chance of causing infection and disease (Finlay & Falkow, 1989). At one end of the spectrum are septicaemias caused by organisms of high virulence such as *S. pneumoniae, N. meningitidis, H. influenzae* type b and *E. coli* type K1 which can cause disease in a proportion of non-immune individuals with intact host defence systems (Easmon, 1990). In contrast are the septicaemias involving many organisms of lower virulence which can infect compromised individuals and cause disease but spares those with intact host defences. Indeed, probably any microorganism possessing the capacity to survive in humans will occasionally cause disease in compromised individuals and behave as an opportunistic pathogen (Finlay & Falkow, 1989).

Minimal requirements for pathogenicity are: 1) ability to enter the host; 2) survive and multiply in the host; 3) resist or avoid host defences; 4) cause damage to the host (Smith, 1977; Hammond *et al*, 1984; Falkow, 1990). Microbial factors responsible for these processes

are the determinants of pathogenicity or virulence factors, and include both cell-associated and extracellular products (Smith, 1977). Although microbial pathogenesis is diverse and multifactorial, common themes of microbial pathogenesis have evolved in distinct bacterial species because of their need to overcome common host and environmental barriers (Falkow, 1990).

The interaction of microbial factors with host mediation systems results in a profound array of pathophysiological sequelae (Ryan, 1985) as shown in the related clinical conditions of septicaemia and septic shock. Although a number of virulence factors participate in the pathogenic process, bacterial endotoxic LPS has been implicated as the major contributory factor to the development of septic shock (Morrison & Ryan, 1987).

4.1 COLONIZATION AND MULTIPLICATION

The sequence of events which may ultimately lead to septic shock begins with the nidus of infection consisting of an abscess, peritonitis, pneumonitis, cellulitis, or another focus (Parrillo *et al*, 1990). It is the ability of bacteria to adhere to and colonize mucosal surfaces which is often the first major stage in the development of infection (Williams & Tomas, 1990). Some microorganisms multiply at and remain on the surface whilst others use attachment as the first essential step before proceeding to deeper tissue or other locations such as the blood stream (Finlay & Falkow, 1989).

Many microbes express several distinct and alternative means of cell attachment, some of which may be expressed under different environmental and host conditions or perhaps at different host surfaces

(Falkow, 1990). *E. coli*, for example, can express several distinct types of fimbriae or adhesins encoded by distinct regions on the chromosome and plasmids (Uhlin *et al*, 1985; Low *et al*, 1987). Many species of Enterobacteriaceae possess the common pili or type 1 fimbriae which enables them to bind to D-mannosyl residues on eukaryotic cells (Clegg & Gerlach, 1987). Although their role in the pathogenesis of infection has been difficult to discern (Freter & Jones, 1983), it is thought they play a significant role in *E. coli* colonization of the urinary tract and in colonization of the large bowel (Finlay & Falkow, 1989). Other cell surface macromolecules such as LPS, capsule and outer membrane proteins could also contribute to the adhesive process. Non-fimbrial adhesins have been shown to surround bacterial cells like capsule (Ørskov *et al*, 1985). Indeed, Kröncke *et al* (1990a) demonstrated the coexpression of these adhesive protein capsules with the K polysaccarides of two pathogenic *E. coli* strains forming a composite capsule.

The host represents an environment of marginal conditions where multiplication constitutes a formidable task for the microbe (West & Apicella, 1985). Competition with the host for a number of essential nutrients appears to be a major factor determining growth rate, with iron assuming an integral role (Weinberg, 1978). To multiply in extracellular body fluids, Gram-negative pathogens must compete for iron associated with host iron-binding glycoproteins, transferrin and lactoferrin (Williams, 1988). A common feature of enterobacteria under conditions of iron deprivation is the production of high molecular mass outer membrane proteins (Williams & Tomas, 1990). These act as receptors for the low molecular mass iron chelators (siderophores) such as enterobactin and aerobactin, also produced under iron restricted

conditions.

The negatively charged nature of the LPS molecule is also thought to be responsible for the sequestration of positively charged ions and molecules. These may be taken up and used in essential metabolic processes, offering bacteria a distinct advantage during *in vivo* growth.

4.2 RESISTANCE TO HOST DEFENCES

The normal host defence mechanisms pose the greatest threat to the survival of an invading pathogen. The maintenance of Gram-negative bacteria within the host entails the evasion of a diverse array of defences that include complement-mediated bacteriolysis, uptake and killing by phagocytes as well as cell-mediated immune mechanisms (Cross, 1990).

Bacterial capsules have long been associated with virulence properties (White, 1938), although only relatively recently have the mechanisms involved been elucidated. The capsules of many Gram-negative bacteria have been shown to confer resistance to phagocytosis. Most capsular polysaccharides are hydrophilic and confer a negative charge on the bacterial cell, characteristics intrinsically antiphagocytic in their effect (Moxon & Kroll, 1990). In the absence of factors which facilitate contact between the bacterium and phagocytic cell and which modify the hydrophilic bacterial surface, phagocytic ingestion of capsulate organism is a very slow and inefficient process. This was highlighted by Mehta *et al* (1988) who demonstrated that rough mutants of Gram-negative bacilli, lacking either capsule or O-antigen were more hydrophobic than the smooth parent strains and more readily ingested.

The K antigen also plays a crucial role in protecting the organism from opsonophagocytosis (in the absence of specific antibodies) and complement mediated lysis. Triggering the antibody-independent alternative pathway depends on the association of C3b (the cleavage product of the third complement component) and factor B to form C3bBb, a C3 convertase which amplifies C3b. Activation of the alternative pathway is triggered by many surface-exposed polysaccharides such as LPS (Marcus *et al*, 1971). The sialic acid polysaccharides of *E. coli* K1 and group B streptococci, however, favour the inactivation of C3b (Stevens *et al*, 1978; Edwards *et al*, 1982). Their strategy is to exploit the competition between factor B which promotes amplification, and factor H, a major regulatory protein of the alternative pathway which terminates it. This enhanced affinity of factor H for cell bound C3b leads to the breakdown of the amplification loop. A similar mechanism of limiting complement deposition has been described for capsules lacking sialic acid (Cross, 1990).

Capsular polysaccharides may also provide virulence by steric mechanisms. The K1 capsule of *E. coli* is thought to mask underlying structures on the bacterial cell surface which activate the alternative pathway (Horwitz & Silverstein, 1980). Alternatively, a capsule may function by preventing the binding of opsonic antibody to its cell wall antigen or prevent the recognition of opsonins by the phagocytes (Easmon, 1990).

Many capsular polysaccharides are poor immunogens including the K1 and K5 capsules of *E. coli* (Cross, 1990). The sialic acid of K1 and desulpho-heparin of K5 mimic important host molecules of mammals (Vann *et al*, 1981; Finne, 1982) allowing them to avoid immune recognition.

Together with other means of resisting host defences it is not surprising that K1- and K5-encapsulated *E. coli* are the most common serotypes found in extraintestinally invasive infections (Cross *et al*, 1984). Other potential strategies of capsules include the shedding of capsule from the bacterial surface to remove attached host factors, or for nullifying the functional role of circulating host factors such as type-specific antibodies (Moxon & Kroll, 1990). It is also proposed that bacterial capsules may exert an effect on the cellular immune response (Cross, 1990).

The LPS phenotype also assumes an important role in the resistance of bacteria against host defences. The O-polysaccharide of some phenotypes have been shown to prevent phagocytosis or complement mediated lysis or both (Williams *et al*, 1983; 1986; Schiller, 1988). In non-immune serum, rough mutants lacking O-antigen are more readily phagocytosed or lysed by complement than O-antigen bearing strains. A number of workers have also established that serum resistant strains often possess longer O-polysaccharides than their serum sensitive counterparts (Goldman *et al*, 1984; Ciurana & Tomas, 1987; Tomas *et al*, 1988). However, sugar composition and structure have also been considered (Rozenberg-Arzka *et al*, 1986; Jiminez-Lucho *et al*, 1987). While the exact mechanisms of resistance are unknown, it is speculated that LPS prevents lysis by activating complement at a site distant from the cell surface. Evidence of exogenous LPS protecting organisms from the bactericidal activities of serum complement, perhaps by its activation and depletion has also been reported (Tanamoto *et al*, 1984; Vukajlovich, 1986).

The ability of a bacterium to resist host defence mechanisms is clearly

emerging as a multifactorial phenomenon, involving both LPS, capsule and other factors. Vermeulen *et al* (1988) showed that the LPS phenotype or presence of a capsule by itself is insufficient to explain a strain's sensitivity to serum bacteriolysis. A comparison of two *E. coli* K1 strains of similar levels of K1 expression demonstrated that the strain with R-LPS was killed quicker than its smooth equivalent. Indeed, a close functional relationship is thought to exist between LPS and capsular surface moieties in their roles as virulence determinants, suggesting the existence of bacterial clones of common origin (Ørskov *et al*, 1976; 1990), (Section 2.5). For example, the K1 capsule is significantly associated with LPS phenotypes (rough and part rough) which are poorly able to resist serum bacteriolysis in the absence of capsule (Gemski *et al*, 1980; Cross *et al*, 1984). However, K5 non-capsulate mutants of smooth *E. coli* O6:K5 retain serum resistance (Cross *et al*, 1986). A comparison of virulence of K1 and K5 capsulate strains with non-capsulate mutants in a neonatal rat model of infection showed significant differences in LD50 between K1 capsulate parent and mutant not observed for the K5 capsulate parent and mutant (Kim *et al*, 1986). Thus, the roles of K1 and K5 capsules appear to be different, and the phenotypes of the associated LPS to be an important variable.

4.3 PATHOPHYSIOLOGICAL CONSEQUENCES OF SEPTICAEMIA

LPS as a toxin

For more than a century many of the pathological sequelae of Gram-negative sepsis have been known to be associated with a heat-stable toxin bound to the cell wall. Structural analysis of endotoxin was initiated by Boivin & Mesrobeanu (1933) who enriched the endotoxic activity from bacterial cell walls. However, it was not until the early nineteen fifties that Westphal & Lüderitz (1954) started to

investigate the chemical nature of endotoxin in greater detail, aiming to purify and identify the fever-inducing principle, to determine its chemical structure, and to understand its mechanism of action in higher animals (Westphal & Lüderitz, 1954).

The biologically active lipid A component of LPS is now well recognized as the primary toxiphore. Evidence for the crucial role of lipid A in LPS-mediated effects was highlighted when the biological activities of both natural and synthetic preparations were found to be identical (Galanos *et al*, 1985b). Although the polysaccharide portion lacks intrinsic endotoxic activity it has a key role in modulating lipid A activity by: rendering the toxic part of the molecule soluble, thereby facilitating its biological interaction with host cells; optimizing the size of micellar aggregates of LPS, and by masking key determinants on the lipid that may be necessary to trigger specific biochemical reactions (Morrison *et al*, 1985; Ryan, 1985). The endotoxic activity of lipid A is thought not to be determined by a single substituent in the sense of a "toxophore group" but by its unique overall conformation (Brandenburg & Seydel, 1984; Rietschel *et al*, 1984a). Indeed, the number of fatty acids within the lipid A, their position, perhaps their chain length and the phosphate content appear to be essential determinants of LPS's endotoxicity (Kanegasaki *et al*, 1986; Cady *et al*, 1989; Loppnow *et al*, 1989).

Although LPS induces acute pathophysiological manifestations reflecting those seen during Gram-negative septicaemia (Ryan, 1985), its role in infection lethality has often been questioned. Parant *et al* (1977) demonstrated that endotoxin-resistant mice (C3H/HeJ) were highly susceptible to lethal infection with Gram-negative microorganisms.

However, despite the uncertainty regarding the proposal that LPS represents the only pathological determinant in Gram-negative bacteraemia, they remain important factors responsible for many of the pathophysiological activities accompanying infection (Ryan, 1985).

Presentation of LPS *in vivo*

LPS activity is markedly influenced by its mode of presentation *in vivo* during an infection where it may assume a number of biochemically or physiochemically distinct forms (Pollack *et al*, 1989). The toxicity of LPS is expressed upon its release from the bacterial cell surface and exposure of the lipid component, and not while it forms an integral component of the outer membrane of intact organisms. Circulating endotoxin probably never occurs as free LPS, but remains strongly associated with an outer membrane protein component when it detaches from the bacterial outer membrane (Hitchcock & Morrison, 1984; Poxton *et al*, 1985; Killion & Morrison, 1986). Formation of these outer membrane complexes imparts a significant increase in LPS toxicity (Straus, 1987; Straus *et al*, 1988). Released LPS molecules from organisms may also form micelles through hydrophobic interactions between lipid A units.

LPS specifically interacts with a number of host components (Brade *et al*, 1988). Of particular importance is the interaction of LPS with serum lipoproteins, especially high density lipoprotein (HDLP), which have been shown to bind rapidly to both circulating free LPS and that contained in membrane fragments (Ulevitch & Johnston, 1978; Munford *et al*, 1982; Munford & Dietschy, 1985). LPS-HDLP complexes have been demonstrated to be much less active than unbound LPS in a number of assays of biological activity (Ulevitch & Johnston, 1978; Warren *et al*,

1986). Cavaillon and colleagues stated that in the process of binding to LP, LPS may be rendered less toxic through a mechanism of decreased ability to induce monocytes and macrophages to release cytokines possibly due to an altered interaction at the cell surface (Cavaillon *et al*, 1990). The significance of LPS-lipoprotein complexes was highlighted when it was proposed they are the predominant form which circulates in the bloodstream (Mathison & Ulevich, 1981).

The binding of LPS to HDLP is thought to be a critical event determining the rate of removal of LPS from the plasma (Ulevitch *et al*, 1979). LPS which do not bind to HDLP appear to leave the circulation rapidly, whereas bound LPS have a prolonged half-life in plasma, and are taken up slowly by tissues (Munford & Dietschy, 1985). The binding of LPS to HDLP, however, appears to be of minor importance in animals having homologous antibodies to LPS. Munford & Dietschy (1985) suggested that antibodies to LPS inhibit LPS-HDLP binding, opsonize both LPS and preformed LPS-HDLP complexes and increase their uptake by phagocytic rich tissues such as the liver and spleen.

There is considerable evidence that the binding of LPS to lipoprotein is altered in inflammatory serum. Lipopolysaccharide binding protein (LBP), a trace protein that binds to lipid A may control the response to LPS under physiological conditions by forming high affinity complexes with LPS that bind to monocytes and macrophages (Schumann *et al*, 1990).

The mediators of septicaemia

Although LPS initiates sepsis, it indirectly affects host tissues by triggering a cascade of biological mediators. LPS is well recognized

as a potent activator of both the humoral and cellular mediation systems, inducing a wide spectrum of acute pathophysiological manifestations the same as those seen during Gram-negative septicaemia and septic shock (Ryan, 1985). These include fever, hypotension, disseminated intravascular coagulation (DIC), and irreversible shock (Morrison & Ryan, 1987).

Whilst the precise cellular and molecular mechanisms whereby LPS triggers these events have not been elucidated, mononuclear phagocytes (monocytes and macrophages) appear to play a central role (Freudenburg *et al*, 1986; Morrison & Ryan, 1987). LPS induces many responses from macrophages, among which are the development of an enhanced respiratory burst (Pabst & Johnston, 1980) and the secretion of inflammatory mediators, including prostaglandins, colony-stimulating factors (Thorens *et al*, 1987) interleukin-1 (IL-1) (Dinarello, 1984) and tumour necrosis factor α (cachectin) (TNF) (Männel *et al*, 1980). The functional inter-relationships of these mediators are complex, since some of them modulate the synthesis or activities of others (Morrison & Ryan, 1987). The fact that endotoxin could activate a series of inflammatory mediators suggested one or more of these intermediators was responsible for the lethality of LPS. Recent studies have focused attention on such cytokines as IL-1 (Weinberg *et al*, 1988), IL-6 (Hack *et al*, 1989; Waage *et al*, 1989), and TNF in particular.

Strong evidence suggests that TNF is a major endogenous toxin in the pathogenesis of endotoxic shock. The central role of TNF as a mediator of LPS-induced injury gained prominence when it was shown to reproduce many of the pathological features of shock (McCutchan & Ziegler, 1983; Remick *et al*, 1987; Mathison *et al*, 1988). Antibodies to TNF were also

demonstrated to confer protection of mice from LPS (Beutler *et al*, 1985) and baboons in an *E. coli* challenge model (Tracey *et al*, 1987). Anti-TNF antibodies reduced the IL-1 and IL-6 responses in this latter study, suggesting an interdependence of these cytokines (Fong *et al*, 1989). Despite these findings, investigations by Neilson *et al* (1989), Silva *et al* (1990a) demonstrated that TNF is necessary but not sufficient to cause death in septic shock, whilst Ciancio *et al* (1991) suggest synergism between TNF and endotoxin (Rothstein & Schreiber, 1988) may be a major component in the pathophysiology of TNF and endotoxin induced shock. Indeed, shock probably results from sequential and synergistic interactions between TNF and a number of biological mediators (Neilson *et al*, 1989; Mózes *et al*, 1991). Proposed candidates include complement (McPhaden & Whaley, 1985), endorphins (Faden & Holaday, 1980), arachidonic acid metabolites (Flohé & Giertz, 1987), and platelet activating factor (Braquet *et al*, 1987). The development of endotoxic shock during Gram-negative septicaemia is thought to occur after the generation of LPS-hyperresponsive macrophages by a sublethal infection (Matsuura & Galanos, 1990).

The relative importance of various mediators is thought to vary with different host responses or bacterial virulence factors including differing patterns of mediator release (Silva *et al*, 1990a). Additionally, individual cytokines may preferentially mediate different aspects of LPS effects. For example, TNF might mediate lethality (Freudenburg *et al*, 1986) or tissue injury (Beutler *et al*, 1985) whereas IL-1 may activate lymphocytes (Dinarello, 1989).

The activation of the complement pathways represents another critical event predisposing the host to septic shock (Goldstein, 1985; McPhaden

& Whaley, 1985). Activated components of complement via the classical and alternative pathways (Morrison & Kline, 1977; Cooper & Morrison, 1978) are potent agents for the stimulation of many of the cellular systems involved in host responses to LPS (Morrison & Ulevitch, 1978). The release of the anaphylatoxins C3a and C5a stimulates the release of histamine which in turn can result in vasodilation. These molecules therefore have the potential, if activated in sufficient quantity, to produce hypotension.

Endotoxin produces marked effects on the coagulation system, and septicaemia often results in DIC (Ryan, 1985). LPS can have fibrinolytic effects through the action of tissue-plasminogen activator and plasminogen-activator inhibitors, which can result in DIC. The classic model of endotoxin-induced shock, the Shwartzman reaction, first suggested that endotoxin perturbs the coagulation system. One of the consequences of DIC, is a haemorrhagic state with profuse bleeding from mucous membranes.

The undoubted detrimental reactions for the host are the result of the over stimulation of host mediator production with an excess of endotoxin (Brade *et al*, 1988). Thus, the release of large amounts of various mediators into the bloodstream profoundly affect the peripheral and pulmonary vascular system, whilst some affect the myocardium itself (Parrillo *et al*, 1990). The vascular and myocardial abnormalities combine to give a generalized cardiovascular insufficiency. Respiratory and renal dysfunction may also be produced by the action of endotoxin. Consequently, septicaemia is a common cause of the adult respiratory distress syndrome (Kaplan *et al*, 1979; Fowler *et al*, 1983). Progression to the later stages of septic shock during severe infec-

tions is commonly characterized by unresponsive hypotension and organ failure.

In contrast to the detailed knowledge on the potential target cells responsive to LPS and the mediators produced by these cells in response to LPS, the mechanisms by which LPS initiates host-cell responses is poorly understood. The identification of relevant LPS receptors on cells of the immune system reviewed by Morrison (1989) is integral to the understanding of endotoxic pathophysiology. Schumann *et al* (1990) proposed a model of LPS induced monocytic stimulation involving the formation of high affinity complexes between LPS and LBP (Tobias *et al*, 1986; 1989). These subsequently interact with CD14, a monocytic differentiation antigen possibly leading to the induction of appropriate transmembrane signals (Wright *et al*, 1990). The ligated CD14 may directly trigger the synthesis of cytokines such as TNF, or indirectly via the production of other proteins.

4.4 HOST RESPONSE TO ENDOTOXIN AND ITS BENEFITS

In vivo, LPS is detoxified by both intravascular antibody and complement mediated mechanisms, and the phagocytic cells comprising the reticuloendothelial system (Skarnes, 1985). Ultimately, most of the LPS seems to be processed by the liver, initially by the liver macrophages, the Kupffer cells. Whilst it is evident that some of the biological activities of LPS are harmful to the host, others appear to be beneficial (Morrison & Ryan, 1987; Brade *et al*, 1988). The beneficial effects of endotoxin are thought to reflect physiological activities of endotoxin being released from Gram-negative bacteria of the gut flora.

LPS is a non-specific B-cell mitogen and has an immunological adjuvant effect for other bacterial antigens. Sublethal Gram-negative infection or LPS administration also induces an increased tolerance to larger doses that usually precipitate a toxic or lethal response (McCuskey *et al*, 1987; Vuopio-Varkila *et al*, 1988). The mechanism of early phase tolerance developed by Greisman & Hornick (1973) is complex, involving a direct endotoxin-cell interaction which inhibits synthesis and/or release of endogenous pyrogen. Recent reports, however, suggest that TNF and IL-1 productions are involved in the induction of early-phase endotoxin tolerance (Fraker *et al*, 1988; Sheppard *et al*, 1989). The ability to produce these, and possibly other mediators in response to LPS may be an important defence mechanism. The protection observed during late-phase tolerance correlates with the presence of serotype-specific antibodies following an immune response to the O-antigen of administered endotoxin (Johnston & Greisman, 1985).

The fine balance existing between an appropriate protective response and over activation of the immune system is markedly influenced by certain predisposing factors discussed in section 1.4. In an evolutionary view of endotoxin, Legrand (1990) proposed that while LPS serves a critical function for Gram-negative bacteria, animals use this cell surface component as a signal for the endotoxin response system. Although responses to endotoxin are commonly classified as being either beneficial or toxic, some of the toxic effects include both protective effects and those which are harmful when taken in excess. However, the unequivocally harmful effects of endotoxin such as free radical damage, DIC, hypotension and death results from this excessive activation of a remarkably well adapted defence system.

CHAPTER 5

TREATMENT OF SEPTICAEMIA AND ENDOTOXAEMIA

5.1 ANTIMICROBIAL THERAPY

The crucial stage in the clinical management of septicaemic patients remains the identification of the responsible bacterial pathogen and determination of its susceptibility to antimicrobial agents (Wood, 1990). Rapid institution of an empirical regimen that includes appropriate therapy on clinical diagnosis of the causative organism(s) has been associated with improved survival and decreased frequency of shock (Kreger *et al*, 1980b; Bryan *et al*, 1983). Prior to identifying the causative organism it is essential that broad-spectrum antibiotic coverage be initiated (Kreger *et al*, 1980b; Parrillo *et al*, 1990). The antimicrobial agents selected for initial therapy are tailored to the individual patient after considering the presumed infecting organism, its likely sensitivity pattern and host factors which may influence the impact of infection on the individual, or the hazards of some agents (Wood, 1990). Depending on the clinical response and results of blood culture and sensitivity testing, these regimens may need to be revised. Initial therapy is often based around the use of a combination of antimicrobials that include an aminoglycoside with a penicillin or second-generation cephalosporin plus metronidazole - if an anaerobic sepsis is suspected (Smith, 1990; Wood, 1990). If previous antibiotics have been given, or the patient is immunosuppressed, a third-generation cephalosporin is frequently used (Geddes, 1988). Although the use of combinations of antibiotics is widely adopted to cover a broad range of potential pathogens, it is now possible to use single antibiotics having broad-spectrum activity such as the 4-fluoroquinolones, notably

ciprofloxacin (Smith, 1990). Despite the introduction of new antimicrobial agents, those with a proven "track-record" continue to be used by clinicians (Smith, 1990).

The frequent failure of antibiotics to ameliorate infections causing septicaemia is in part explained by their ineffectiveness, against the toxic effects mediated by endotoxins (Fomsgaard, 1990). Thus, although antibiotics are of value during the initial bacteraemic stage, they are ineffective later in the course of illness. Evidence has also revealed that antibiotic therapy can encourage endotoxin release during therapy, thereby aggravating the clinical symptoms of disease (Cohen & McConnell, 1985; McConnell & Cohen, 1986). Studies have demonstrated that the rate of liberation of endotoxin is different for antibiotics of different classes, despite similar bactericidal activities (Shenep & Mogan, 1984; Shenep *et al*, 1985b). Despite being unable to prevent continued influx of bacteria into the bloodstream, bacteriostatic antibiotics were however shown not to induce endotoxin release. Indeed, Kreger *et al* (1980b) reported a higher survival rate in patients treated with bacteriostatic antibiotics compared to those treated with bactericidal agents. Further support for the detrimental effects of antibiotics was highlighted by Shenep *et al* (1988) who established a marked increase in both bacterial-bound and free endotoxin levels in the plasma of patients with septicaemia during antibiotic therapy.

Polymyxin B, a polycationic antibiotic which binds to LPS is known to block many of its biological activities *in vitro* (Craig *et al*, 1974; Spear & Teodorescu, 1984). It has also been shown to protect against experimental models of endotoxaemia (Rifkind, 1967; Baldwin *et al*,

1991) and septicaemia (Flynn *et al*, 1987), although its high toxicity is thought to limit its therapeutic potential. The protective mechanism afforded by polymyxin B against LPS-induced toxicity is proposed to be the inhibition of TNF production (Stokes *et al*, 1989).

Despite important advances in the development of antimicrobial agents septic shock remains a serious disease with high mortality. Since clinical results from antimicrobial therapy appear to be maximized, considerable interest lies in the development of therapeutic strategies which neutralize or inhibit the key toxins and mediators of septicaemia.

5.2 ANTI-ENDOTOXIN ANTIBODIES

Considering the central role of endotoxin in Gram-negative septicaemia, and the success of immunotherapy in the treatment of other bacterial diseases involving the release of toxins, it appears that antibodies to LPS might be of some value in the treatment of septicaemia. Despite important advances on the protective role of anti-LPS antibodies over the last decade, the specificity and the type of anti-LPS antibody required for protection, as well as the mechanism(s) of protection, have not been completely defined (Ziegler, 1988).

Antibodies to serospecific determinants are thought to opsonize LPS and whole bacteria, and to provide an additional bactericidal effect on intact Gram-negative bacteria (Pennington & Menkes, 1981; Kauffmann *et al*, 1986; Ziegler, 1988). Previous studies have demonstrated the ability of polyclonal or monoclonal anti-O-polysaccharide antibody to protect against experimental bacterial infections (Kaijser & Ahlstedt, 1977; Colwell *et al*, 1984; Dunn *et al*, 1985; Pluschke & Achtman, 1985;

Zweerink *et al*, 1988). Active immunization of animals to produce high levels of serospecific antibodies has also conferred protection (Young, 1972; Cryz, *et al* 1984; 1985).

Although O-antigen specific antibodies appear to be effective, it is not feasible to produce a polyvalent antiserum against all potential pathogens in human septic shock (Ørskov *et al*, 1977; Ziegler, 1988). However, since approximately 10 O-serotypes account for roughly 70% of *E. coli* bacteraemic isolates (McCabe *et al*, 1978; Cross *et al*, 1984; Ørskov & Ørskov, 1985) the possibility has been raised of developing an O-serospecific vaccine against this leading cause of nosocomial Gram-negative septicaemia. Preliminary work by Cryz *et al* (1990) led to the production of a non-toxic *E. coli* O18 O-polysaccharide (O-PS)-toxin A and O-PS-Cholera toxin conjugate vaccines which afforded protection against a challenge from the homologous strain when passively transferred to mice. Their safety and immunogenicity in humans has also been established, providing impetus for the development of further conjugate vaccines against other important *E. coli* serotypes (Cryz *et al*, 1991). Many high risk patients are immunocompromised in some manner, and therefore not ideal candidates for active vaccination. A more promising use of such vaccines includes the proposed preparation of a hyperimmune gammaglobulin for intravenous use (IVIG) from the plasma of immunized healthy donors (Cryz *et al*, 1991). The preparation of such an IVIG needs to consider that the majority of nosocomial Gram-negative septicaemias are caused by *E. coli*, *P. aeruginosa* and *Klebsiella* species (McGowan *et al*, 1975; Bryan *et al*, 1983; Easmon, 1990). A polyvalent IVIG to these three pathogens would increase the spectrum of activity thereby lending itself for use prophylactically or perhaps immunotherapeutically. Multivalent *P. aeruginosa* O-polysaccha-

ride conjugate vaccine and *Klebsiella* capsular polysaccharide vaccine have already been prepared and shown to be safe and immunogenic in humans (Granström *et al*, 1988; Cryz *et al*, 1989). The possibility of combining these existing vaccines with an *E. coli* component is particularly attractive and awaits future developments (Cryz *et al*, 1991).

Another approach to the development of a protective agent against septicaemia includes the use of antibodies directed towards the conserved regions of the LPS molecule, the core-glycolipid. It was postulated that these common determinants might stimulate widely cross-reactive antibodies to LPS which could inhibit its endotoxic activities (Chedid *et al*, 1968). Great emphasis has been placed on characterizing antisera against rough mutants lacking O-antigens, in particular the R mutants of *E. coli* O111:B4 (J5) and *S. minnesota* (R595), which express only lipid A and a portion of the core oligosaccharide as their LPS (McCabe *et al*, 1972; Fuller *et al*, 1973).

Polyclonal antiserum raised against rough mutant bacteria has been shown to protect animals from a wide variety of Gram-negative bacteria (Ziegler *et al*, 1973a & b; Young *et al*, 1975; Marks *et al*, 1982) and endotoxins (Braude & Douglas, 1972; Braude *et al*, 1973; Davis *et al*, 1978). In addition, the discovery of naturally occurring antibodies to endotoxin-core structures in animals, including man, led to a series of retrospective studies in patients with Gram-negative bacteraemia. These demonstrated that patients with high levels of anti-core antibody at the time of presentation had a reduced incidence of septic shock and lower mortality (McCabe *et al*, 1972; Pollack *et al*, 1983). Such observations prompted clinical studies with antiserum obtained by

immunizing volunteers with the rough mutant *E. coli* J5. Ziegler *et al* (1982) commenced a double-blind, randomized, controlled trial of polyclonal anti-J5 antiserum versus control, non-immune serum in patients with established Gram-negative bacteraemia. Mortality was reduced overall from 39% in control patients to 22% in recipients of anti-J5 antiserum (p0.011). In the subgroup of patients with severe shock, the results were even more convincing; 77% of control patients died compared with 44% of the anti-J5 group (p0.003). A subsequent clinical trial by Baumgartner *et al* (1985) successfully demonstrated the protective prophylactic use of anti-J5 antiserum in high-risk intensive-care patients.

The protective element of antisera against rough mutant bacteria has been hypothesized to be cross-reactive anti-core-glycolipid antibodies which neutralize the harmful effects of endotoxins. Indeed, many believe its effects depend primarily upon IgM antibody neutralizing the toxic properties of LPS, perhaps by steric hinderance, although enhanced bacterial clearance may also play a part (Ziegler *et al*, 1973a; Young *et al*, 1975; Sakulramrung & Domingue, 1985). A number of *in vitro* and *in vivo* studies have however failed to show these cross-reactive and protective properties (Ng *et al*, 1976; Greisman *et al*, 1978; 1979; Gigliotti & Shenep, 1985; Trautmann & Hahn, 1985). Even in studies showing protection, it has been difficult to demonstrate that the antisera contain antibodies which cross-react with LPS from smooth strains, and establish a close relationship between protection and the levels of core-glycolipid antibodies (Ziegler *et al*, 1982; DeMaria *et al*, 1988). This inability to measure the specific protective antibody in polyclonal antiserum has resulted in uncertainty regarding its protective mechanism (Baumgartner & Glauser, 1987; Baumgartner *et al*,

1991). Much uncertainty also relates to the great variation in the protective properties of pre-immune sera owing to the presence of naturally occurring anti-LPS antibodies, and vaccination procedures giving rise to considerably different titres.

The presence of high affinity antibodies to type specific immunodominant core LPS determinants are also thought to obscure cross-reactive antibodies in antisera to rough mutants (Baumgartner *et al*, 1987). Since considerable amounts of immunogen-specific antibodies may be stimulated by novel epitopes of R-submutant LPS it is possible the cross-reactive anti-LPS activity in R-LPS-immune sera has been over-estimated (Baumgartner *et al*, 1987; Cross *et al*, 1989; Barclay, 1990).

In addition to the hypothesis attributing cross-protection of antisera to antibodies, protection may be conferred by small amounts of contaminating endotoxin, which might cause tolerance (Chong & Huston, 1987), or polyclonal B-cell activation (Siber *et al*, 1985), or other non-antibody proteins present in the antiserum (Brade & Brade, 1985). Warren *et al* (1991) established the requirement of serum for the optimal interaction between smooth (S)-LPS and IgG fractions from J5 antiserum. They suggested these results may explain discrepancies shown in previous *in vivo* and *in vitro* studies. Concern has also been raised over the suitability of the J5 mutant as an immunogen. The core structure of *E. coli* J5 appears to be distinct from those of other bacteria and exhibits greater heterogeneity, thus limiting the cross-reactive potential of antibody raised against J5 (Fuller *et al*, 1973; Appelmelk *et al*, 1986a). Antisera raised against rough mutants continue to demonstrate protection (Baumgartner *et al*, 1987; Calandra *et al*, 1988) although it is not feasible this form of antisera will

ever become available on a large scale (Johns *et al*, 1983).

The potential use of naturally occurring anti-LPS antibodies in humans as an immunotherapeutic agent has also been investigated. The importance of these antibodies in the defence against infections has long been established by clinical studies relating the outcome of infection in patients to titres of specific and, in particular, cross-reactive antibodies prior to bacteraemia (McCabe *et al*, 1972; Pollack *et al*, 1983). A number of further complementary reports have established a depression of antibodies to the LPS molecule in patients with Gram-negative septic shock which, together with rising or constant endotoxaemia are associated with early mortality (Nys *et al*, 1988; Peter *et al*, 1979; Schedel, 1988). Barclay *et al* (1989) demonstrated depression of intrinsic serum IgG antibody levels to structures in the inner core region of LPS during shock, while those to the outer core or O-polysaccharide region remained unaltered. Temporal variation in levels of individual anti-LPS antibodies were also related to variations in endotoxaemia, since depressions of certain anti-LPS antibodies occurred immediately before peaks of endotoxaemia, and recovery of these anti-LPS antibody levels preceded fall in endotoxin.

Several reports have emphasized the importance of anti-lipid A (Cerra, 1987; Schedel, 1988) or Re-LPS and lipid A antibodies (Schellekens *et al*, 1989) in septicaemia, whilst others suggest that cross-reactive IgG antibodies to a variety of epitopes in the endotoxin are prevalent in normal human sera and can be depressed during septicaemia (Barclay & Scott, 1987; Wessels *et al*, 1988). Thus the passive administration of antibodies of appropriate specificities during endotoxaemia may mediate some clinical benefit by reducing the level or duration of endotoxaemia

and its subsequent clinical effects.

The use of standard IVIG has been shown to prevent the acquisition of bacterial infections in hypogammaglobulinaemic patients (Pirofsky, 1984). Although evidence exists for its potentially beneficial effect in the treatment of septicaemic patients (Collins *et al*, 1986; Baumgartner & Glauser, 1987; De Simone *et al*, 1988) there is insufficient justification for its widespread use (Cohen, 1988; Dudley, 1990). Indeed its prophylactic and therapeutic use in clinical trials showed only marginal benefit (Duswald *et al*, 1980; Schmidt *et al*, 1984; Glinz *et al*, 1985; Just *et al*, 1986).

An alternative approach has been the use of hyperimmune IVIG, that is, plasma donors with especially high titres to core antigens. Screening of blood donor populations has revealed the presence of a wide range of anti-LPS antibody concentrations. The small number of individuals showing very high titre anti-LPS antibodies may provide sera for preparation of a therapeutic hyperimmune gammaglobulin (Appelmelk *et al*, 1985; 1987; Barclay & Scott, 1987; Fomsgaard *et al*, 1987; Scott *et al*, 1990). Antibodies to all LPS substructures are simultaneously present in such sera, and screening for one antibody specificity also selects sera containing anti-LPS of other specificities (Fomsgaard, 1990). A broad range of specific as well as cross-reactive anti-LPS antibodies is therefore obtained by the pooling of selected sera. Scott & Barclay (1990) observed multiple reactivity in individual high-titred sera, predominantly attributable to one of two kinds of cross-reactive families of anti-LPS core antibodies: those reactive with both Re-LPS and Rc-LPS (anti-lipid A/KDO), or with Rc-LPS but not Re-LPS (anti-heptose). Therapy with single anti-LPS sera portions is thought

unlikely to possess an appropriate combination of antibody specificities (Gaffin *et al*, 1985). Screening for anti-LPS antibodies also prevents the problems of uneven antibody titres, and risks of vaccination.

The potential of hyperimmune IVIG has been demonstrated in a number of trials. Gaffin *et al*, (1982) screened blood donors for natural antibody to LPS by ELISA using a cocktail of 12 purified endotoxins. Selected anti-LPS rich material was used in an open trial of septic shock complicating obstetric conditions (Lachman *et al*, 1984). Results indicated a reduction in mortality from 47% in the control group to 7% in the treated group ($P<0.01$). The same group has also produced an equine anti-endotoxin by immunizing horses with a pool of endotoxins, which has subsequently been used in a variety of endotoxin implicated conditions (Wells & Gaffin, 1987). Fomsgaard *et al* (1989) demonstrated the antibody mediated neutralization of different biological effects *in vitro* and the protection against lethal toxicity induced by either purified LPS or Gram-negative infection. Protection was independent of the specific anti-LPS titre, suggesting that protective antibodies were a fraction of the total antibody present, possibly directed to less exposed structures in the core-glycolipid region. The anti-LPS IgG was used in a preliminary clinical trial (Fomsgaard, 1990). Intravenous treatment of patients in septic shock was associated with a decrease in serum concentrations of endotoxin and TNF, improvement of the clinical condition, and a decrease in expected mortality from 80% to approximately 50%. An additional report describes seven patients with Gram-negative septicaemia who were given a commercial human IgG preparation shown to have a high titre of antibody to lipid A (Marget *et al*, 1985). Four patients survived and a follow up randomized, double blind study

also showed an improved outcome (Jaspers *et al*, 1987).

Continued investigation of the properties of naturally immune blood donor sera is required for selection of appropriate hyperimmune anti-LPS gammaglobulin for passive immunotherapy of septic shock. The determinations of the exact specificity of the protective antibodies is essential since it will permit the screening of appropriate blood donors for clinical use as well as the production of effective monoclonal antibodies (MAbs). However, this anti-LPS specificity may be different for different LPSs and also for protection against endotoxaemia and bacteraemia (Fomsgaard & Galanos, 1989; Fomsgaard, 1990). The protective mechanisms conferred by anti-LPS immunoglobulins is equally uncertain. The possession of determinants against core and surface structures may act by neutralizing both the toxic-groups responsible for the pathophysiological effects of LPS and opsonization and complement-mediated bacterial killing (Cohen, 1986). Scott & Barclay (1990) established major opsonic activity of natural cross-reactive IgG antibodies binding to the core of the LPS molecule for both rough and smooth strains of Gram-negative bacteria. Such antibodies may perform an important defence mechanism against infection prior to the production of serospecific antibodies.

The relative protective abilities of antibody isotypes (IgG or IgM) is still unresolved (Young *et al*, 1975; Ziegler *et al*, 1982; McCutchan & Ziegler, 1983; Marget *et al*, 1985). This is of particular practical significance since IVIG contains little or no IgM. Previous studies have demonstrated a stronger association of protection with IgG antibodies compared to IgM (Young *et al*, 1975; Fenwick *et al*, 1986). Others have shown that sera from humans or animals deliberately

immunized to produce antibodies to the *S. minnesota* Re LPS core are cross-protective against bacteraemia and endotoxaemia when given passively to animals, but that protection was provided by IgM and not IgG (DeMaria *et al*, 1988; McCabe *et al*, 1988). However, naturally immune IgG from selected blood donors may have a different range of anti-LPS specificities and protective properties from 'Re-immune' IgG or IgM (Barclay *et al*, 1989). Young *et al* (1989) suggested protection seen with different isotypes may be related to the nature of the experimental model. IgG antibodies may appear more protective where infection has been initiated in peripheral tissue or the lung. However, in models of acute bacteraemia, IgM antibodies may be more effective on a molar basis because of their greater number of antigen binding sites. Results of recent and ongoing investigations with anti-core-glycolipid antibodies are anticipated to relate antibody function directly to specificity and isotype. This information will allow the testing in carefully controlled clinical trials, the efficacies of hyperimmune IVIG preparations and MAbs in the treatment of critically ill patients (Baumgartner & Glauser, 1987).

The advent of MAb technology (Köhler & Milstein, 1975) opened up the possibilities of commercial production of those immunotherapeutic agents which overcome some of the drawbacks associated with polyclonal antisera to LPS. The potential of MAb anti-LPS antibodies directed towards the conserved regions of the core-glycolipid has been intensively investigated over the last decade for immunotherapy of septicaemia. A number of studies have demonstrated broad cross-reactivity of MAbs, indicating that commonly shared antigenic determinants are present in the LPS of many unrelated species (Mutharia *et al*, 1984; Nelles & Niswander, 1984; Teng *et al*, 1985; Kirkland *et al*, 1986;

Miner *et al*, 1986; Bogard *et al*, 1987; Pollack *et al*, 1989). Most widely cross-reactive MAbs react with the lipid A component of LPS, and are considered the best candidates for cross-protective immunotherapeutic reagents owing to their ability to inhibit the biological activities of LPS (Mayoral & Dunn, 1990; Mehta *et al*, 1990; Cornelissen *et al*, 1991).

The immunotherapeutic potential of these agents has been illustrated by their ability to confer protection in a number of experimental infection and endotoxaemia models (Dunn *et al*, 1985; Teng *et al*, 1985; Coughlin *et al*, 1986; Dunn *et al*, 1986; Ziegler & Teng, 1986; Dunn *et al*, 1988; Priest *et al*, 1989). Other cross-reactive MAbs have however failed to protect against lethal infection, possibly due to their inability to bind to inner-core and lipid A determinants on smooth bacteria (Ward *et al*, 1988; Salles *et al*, 1989; Evans *et al*, 1990; Mandine *et al*, 1990). Thus, the importance of anti-lipid A antibodies in host defence may lie more in their ability to neutralize pathological effects of LPS, than in their ability to protect against bacterial infection. The precise mechanism of inhibition of lipid A mediated biological effects is unknown. Antibody may bind to the biologically active site of lipid A, blocking its activity directly, or antibody may bind to an epitope close to the active site of lipid A such that they interfere sterically with the stimulation of target cells by lipid A (Ward *et al*, 1988; Mehta *et al*, 1990). The binding of antibodies to LPS may also cause a conformational change in the LPS molecule or micelle thereby affecting the interaction of lipid A with receptor cells.

The composition and number of long-chain fatty acids attached to lipid

A backbone are critical determinants of toxicity (Brade *et al*, 1988), suggesting the important role played by these hydrophobic elements of lipid A in the mediation of cellular responses. Indeed, Cornelissen *et al* (1991) demonstrated the neutralization of lipid A-mediated effects in the presence of a MAb recognizing the hydrophobic elements of lipid A. Failure of MAbs described by Chia *et al* (1989) to inhibit LPS-induced secretion of TNF by macrophages may have been caused by their specificity for the hydrophilic elements of lipid A.

Although a number of core-glycolipid MAbs have been produced, a great deal of controversy exists regarding their epitope specificity, cross-reactivity and immunoprotective potential. Much of this confusion is related to the problems of immune sera, where the specificity of antibodies has been inadequately defined (Barclay, 1990). Many MAbs have been found to be specific for the immunodominant terminal epitopes of their immunogen. If the immunogen is R submutant LPS the cross-reactivity is often restricted to LPS of a similar chemotype (De Jongh-Leuvenink *et al*, 1986; Brade *et al*, 1987b; Pollack *et al*, 1989). Cross-reactivity is also limited by inter and intraspecies differences in covalent core structures (Pollack *et al*, 1989). Lipid A, considered the most conserved structural element of LPS, exhibits considerable phylogenetic variation in polar head group substitution and fatty acid composition (Rietschel *et al*, 1984b). Such variation can introduce intrinsic structural microheterogeneity to the molecule, leading to class related, but not identical lipid A chemical structures (Schwartzer *et al* 1989).

Uncertainty regarding the specificity and cross-reactivity of MAbs is also related to the lack of reliable and reproducible *in vitro* tech-

niques for measuring reactivity of antibodies to heterologous Gram-negative bacteria and LPS. A number of studies have demonstrated that in addition to epitope specificity, cross-reactivity of MAbs is a function of the physical state of bacteria or LPS used in an assay, and the assay itself (Aydintug *et al*, 1989; Pollack *et al*, 1989; Heumann *et al*, 1991). The putative cross-reactive and endotoxic properties of many MAbs to the core-glycolipid are restricted by epitope concealment by O-side chain and outer core sugars, preventing access to these underlying determinants (Nelles & Niswander, 1984; Gigliotti & Shenep, 1985; Miner *et al*, 1986; Pollack *et al*, 1987).

Although the controversies concerning *in vitro* cross-reactivity of LPS antibodies are not resolved, two large clinical trials using MAbs seem to support the concept that anti-LPS antibodies may be protective in clinical practice. The first used a murine IgM MAb designated as E5, directed against the lipid A region of Gram-negative bacterial endotoxin (Harkonen *et al*, 1987). The analysis of data suggests that the difference in overall survival between the treated and untreated groups were not significantly different (Bernard *et al*, 1989). However, the MAb was possibly protective in patients who were not in shock, and treatment was also shown to be beneficial in the resolution of organ failures, regardless of the presence or absence of shock. Murine MAbs have however been shown to be immunogenic in humans (Schroff *et al*, 1985), prompting the production of either chimeric (mixed mouse and human origin) and human MAbs for possible therapeutic use.

The second trial used a human anti-lipid IgM MAb designated as HA-1A or Centoxin ™, produced from a cell line developed by Teng *et al* (1985).

Results indicated that mortality in patients with Gram-negative bacteraemia was reduced by 39% and in patients with septic shock by 42% (Ziegler *et al*, 1991). The antibody also appeared to have a prophylactic effect in preventing the development of symptoms, if administered early enough. However, those patients without positive blood culture did not benefit from the administration of HA-1A. Additionally, the properties of HA-1A are still not fully understood, and its mode of action poorly defined. Overall, these findings illustrate the potential of monoclonal antibody immunotherapy in clinical medicine and supports the continued development of other, perhaps improved antibodies.

5.3 OTHER STRATEGIES

Apart from the use of antimicrobials, early administration of appropriate supportive therapy is also required to influence progression of the condition positively. The intensive-care of septicaemic patients requires rapid therapeutic intervention with appropriate fluids and vasopressors before hypotension becomes evident, together with other cardiac, pulmonary and metabolic support (Wolf, 1982: Young, 1985; Luce, 1987). Despite being useful adjuncts to other therapies, such clinical management of septic shock is merely supportive to the host's defences which must eventually effect a recovery. As the understanding of the pathogenic mechanisms of the illness advances, a greater awareness of the requirements for more effective resuscitation will become apparent.

Avoiding septicaemia and taking measures to minimize the risk of septicaemia are the easiest ways to lower mortality (Dipiro, 1990). These include avoiding the over excessive use of antimicrobials and the

use of invasive medical devices. A reduced frequency of septicaemia has also been achieved for susceptible patients undergoing surgery using the method of selective bowel decontamination (Alcock & Ledingham, 1988; Schmeiser *et al*, 1988; Wiesner *et al*, 1988). This strategy involves the use of oral antimicrobial agents which remove opportunistic aerobic and facultatively anaerobic organisms from the gut. However, the technique is ineffective against the threat of septicaemia from exogenous sources or resistant organisms.

Since the macrophage product TNF appears to be one of the principal mediators of endotoxin induced shock, there is considerable interest in the prospect of the therapeutic use of anti-TNF MAbs against the effects of septicaemia. An advantage of anti-TNF therapy is the potential effectiveness against organisms other than Gram-negative bacteria in the management of shock. Moreover, blocking TNF activity may have uses in treating diseases other than septic shock. To date, there has been uniform agreement on the efficacy of anti-TNF as prophylaxis in experimental models of endotoxic shock (Beutler *et al*, 1985; Tracey *et al*, 1987; Mathison *et al*, 1988). Anti-TNF MAbs have also been effective in infection models (Opal *et al*, 1990; Silva *et al*, 1990b), although a major concern is the apparent lack of protection if antibody is administered after, rather than before bacterial challenge (Silva *et al*, 1990b). The clinical use of anti-TNF antibodies in human patients remains to be firmly established. However, successful phase 1 trials of such an agent have been reported (Exley *et al*, 1990).

Since TNF production is triggered by endotoxin itself, it is postulated that the utility of combination therapy using anti-TNF and other anticytokines with anti-LPS may be the most effective treatment (Cohen,

1989). Anti-LPS MAbs could serve to inhibit the initial progression caused by the septic insult, whilst inhibitors of mediator-induced effects may further limit the deleterious host response. Although cytokines such as TNF and IL-1 are important mediators of Gram-negative shock a greater understanding of the septic shock pathogenic cascade is still required. This may stimulate the development of effective therapies interrupting key steps in the sequence, and reduce the high mortality associated with this disease (Parrillo *et al*, 1990). Given the complexity of the response to LPS it has also been postulated that an antagonist of the initial interaction of LPS with macrophages would have great therapeutic potential (Raetz, 1990). A recent study by Morrison *et al* (1990) demonstrated the protection afforded by a MAb against LPS receptors expressed on murine macrophages and lymphocytes. The immunotherapeutic potential of this or related MAbs remains to be determined. Inactive precursors and synthetic analogues of lipid A have prevented the toxic effects of LPS (Danner *et al*, 1987; Golenbock *et al*, 1987), whilst the non-toxic LPS of *Rhodobacter capsulatus* may provide an attractive alternative in preventing the fatal effects of septicaemia by competing with toxic LPSs (Loppnow *et al*, 1990).

AIMS OF THE THESIS

Endotoxin, primarily the LPS component of Gram-negative bacterial outer membrane, is one of the most important components responsible for the development of endotoxic shock. The objective of this thesis was to investigate the expression of *E. coli* LPS under different environmental conditions and the detection of specific LPSs with MAb probes.

The areas of research studied include:-

1) Characterization of anti-LPS MAbs in a number of assay systems employing different LPS preparations for use as tools for studying the expression and detection of LPS.

2) The use of MAbs in the development of a capture ELISA system for the detection of *E. coli* LPS core and O-types. The underlying objective of this study was to use MAbs for the detection of specific LPS types in the serum of septic patients.

3) Investigation of the expression of LPS on bacteria grown *in vitro*, under environmental conditions mimicking those of an infected host, and on bacteria grown *in vivo*.

4) The effects of subinhibitory concentrations of various antibiotics on the binding of anti-LPS MAbs to *E. coli* and the expression of *E. coli* LPS and outer membrane proteins.

MATERIALS AND METHODS

CHAPTER 1

MATERIALS

1.1 BACTERIA

Bacterial strains

Bacterial strains and their source used in this thesis, are shown in Table 2.

Maintenance of bacterial strains

Bacterial strains were maintained in a lyophilized form. Suspensions of bacteria, prepared from bacterial cultures (Section 2.1) were aseptically aliquoted into sterile glass ampoules and lyophilized (Edwards Modylo freeze dryer, Edwards High Vacuum Ltd., Crawley, Surrey).

Detection of K1 and K5 *E. coli* capsules

The detection or confirmation of K1 and K5 expression was performed by the phage typing method described by Devine *et al* (1990). K1 and K5 specific bacteriophage (designated φK1GS and φK5DG) and their respective propagating strains were obtained from Dr A P Roberts (Department of Medical Microbiology, Charing Cross and Westminster Medical School, London).

1.2 CHEMICALS, MEDIA AND REAGENTS

Chemicals

Unless otherwise stated, all chemicals were Analar grade (BDH Chemicals Ltd., Poole, Dorset). All solutions were prepared in 'Milli-Q' pyrogen-free ultra-pure water.

Table 2. Bacterial Strains

Bacterial Strains		Source
E. coli		
Core R-LPS mutants	R1	Dr H Brade
	R2	Forschungsinstitut
	R3	Borstel
	R4	Germany
	K12	
	018:Krf (isogenic mutant of 018:K1)	Dr A S Cross (see below)
E. coli		
Serotype strains	02:K?	Dr A S Cross
	04:K?	Dept of Bacterial Diseases
	06:K5	Div of Community Diseases
	012:K?	and Immunology
	015:K?	Walter Reed Army Institute of
	016:K1	Research
	018:K1	Washington DC USA
	018:K1⁻ (isogenic mutant of above)	
E. coli		
Clinical, blood culture isolates	018:K5 (226)	Dr A P Gibb
	018:K5 (281)	Dept of Medical Microbiology
	01:K1 (316)	University Medical School
	06:K? (317)	Edinburgh
	0?:K5 (484)	
S. typhimurium		
Core R-LPS mutants	R1542 Ra	Dr I W Sutherland
	R878 Rc	Institute of Cell & Molecular
	R1102 Re	Biology
		King's Buildings Edinburgh
Serotype strain	SL1181	Dr N D Mehta
		St Mary's Medical School
		London
K. pneumoniae		
Serotype strain		Dr I W Sutherland (as above)
P. aeruginosa		
Serotype strain	011	Dr R J Jones
		University of Liverpool

Media

a) **Nutrient agar** was Columbia agar base (Oxoid Ltd., Basingstoke, Hants.).

b) **Iso-sensitest agar (Oxoid)**

a) and b) were prepared by the Medical Microbiology Media kitchen according to manufacturers' instructions. Media was sterilised by autoclaving at 15 psi for 15 min.

c) **Nutrient broth** was Gibco and prepared at the Blood Transfusion Service Protein Fractionation Centre, Edinburgh. The medium was sterilised by ultrafiltration.

d) **Malka minimal medium** was prepared as a modification of the medium of Robert-Gero *et al* (1970) as follows:

Solution A NA_2HPO_4(73.4 mg ml^{-1}), KH_2PO_4 (32.4 mg ml^{-1})

Solution B $MgSO_4.7H_2O$ (20.5 mg ml^{-1})

Solution C 20% w/v glucose

Solution D $FeSO_4$-$7H_2O$ (1.83 mg ml^{-1}) in sterile distilled water to which one drop of concentrated hydrochloric acid (HCl) was added

Solution E $(NH_4)_2SO_4$ (50.0 mg ml^{-1})

All chemicals were from BDH. Solutions were prepared with sterile distilled water and were filter sterilised. All solutions except C were stored over chloroform. To prepare 1 litre of MALKA, 20 ml A, 20 ml B, 20 ml C, 1 ml D and 20 ml E were added to 919 ml sterile distilled water. Modifications of this medium were prepared by the addition of either a 10%, 5%, 1% or 0% volume of the magnesium salt (Solution B) used for the standard minimal medium.

e) **Nitrogen deficient/high carbon medium** was prepared following the

method of Sutherland & Wilkinson (1965) containing: 1 g yeast extract (Oxoid); 1 g casamino acids (Difco technical grade); 10 g Na_2HPO_4; 3 g KH_2PO_4; 0.2 g $MgSO_4.7H_2O$; 11 g K_2SO_4; 1 g NaCl; 0.01 g $CaCl_2$; and 0.01 g $FeSO_4$. The volume was made up to 1 litre with sterile distilled water and autoclaved. To this, 20% w/v filter-sterilised glucose solution was added to give a final concentration of 2% glucose w/v.

f) **Iron depleted medium** was prepared by the addition of 150 μmol. L^{-1} 2,2' dipyridyl (Sigma Chemical Co., Poole, Dorset) to Gibco nutrient broth. Modifications to this medium used in growth experiments are described in the results.

g) **Magnesium/iron depleted medium** was prepared by the addition of 150 μmol. L^{-1} 2,2' dipyridyl to a modification of the Malka minimal medium containing 1% of the normal magnesium salt concentration.

h) **Sheep serum** was obtained from the Moredun Research Institute, Gilmerton Road, Edinburgh. Serum was filter sterilised (0.45 μm pore size) and stored at -20°C. Heat-inactivated serum was prepared by heating at 56°C for 60 min and stored as above.

Lipopolysaccharides

A number of lipopolysaccharides were prepared as described below.

Serum Human serum containing low concentrations of core-glycolipid antibodies for spiking with LPS was obtained from blood donors at the Blood Transfusion Centre, Edinburgh.

Antibiotics

The β-lactam, Ampicillin (Beecham Research Laboratories, Brentford, England); Chloramphenicol (Parke-Davis & Co., Hounslow, London); the

quinolone Ciprofloxacin (Bayer UK, Hayward's Heath, West Sussex), and the aminoglycoside Gentamicin (Parke-Davis & Co.) were used. Antibiotic solutions were prepared according to the manufacturers' instructions immediately before each experiment.

Monoclonal antibodies

All MAbs were obtained from fusions carried out in the Department of Surgery, University Medical School, Edinburgh. MAbs were prepared by fusing spleen cells from immune BALB/c mice with NS-O myeloma cells by standard techniques (Kipps & Hertzenberg, 1986). The reactivity of the MAbs was determined by a LPS-polymyxin enzyme linked immunosorbent assay (ELISA), as described by Scott & Barclay (1987). A summary of the MAbs used and their immunogen(s) is presented in Table 3.

Supernatant fluids of hybridoma cell cultures grown in RPMI 1640 supplemented with 5% v/v fetal calf serum in 150 cm^2 flasks were used throughout unless specified. Cell cultures were grown to maximum cell density and harvested at 50% cell viability. These hybridomas gave yields of approximately 50 $\mu g/ml$.

Mouse MAbs were isotyped using a mouse monoclonal antibody isotyping kit (RPN29) (Amersham International plc, Amersham, Bucks).

1.3 EQUIPMENT

Equipment and apparatus used in the experimental studies are described in the relevant section of the text. Manufacturers' addresses are only cited once.

Table 3. Monoclonal Antibodies

Fusion	MAb	Immunogen	Isotype
27	27.150.3 27.193.3	Cyclic: *P. aeruginosa* PAC605; *E. coli* R1, R2, R3, R4, K12 Heat-killed bacteria	IgG2a IgM
30	30.4.2.8	*E. coli* J5 Lipid A on cytoden beads then J5 and Re595 LPS booster	IgM
40	40.18.7.1	*E. coli* R3 Heat-killed bacteria	IgG3
43	43. 3. 4.8 43. 5. 1.4 43.11. 5.1 43.27.11.2 43.35. 1.4	*E. coli* O18 rough Heat-killed bacteria	IgG3 IgM IgG2a/IgM IgG3 IgG3
184	184.2.5.5	*E. coli* O18:K1$^-$ Heat-killed bacteria	IgG
185	185.1.2.2	*E. coli* O6 Heat-killed bacteria	IgM

CHAPTER 2

EXPERIMENTAL METHODS

2.1 CULTURE OF BACTERIA

All bacteria were cultured at 37°C in air. Bacteria were streaked onto nutrient agar from lyophilized maintenance cultures, resuspended in a small volume of nutrient broth. Subculture of bacteria onto fresh plate media was performed fortnightly for up to three subcultures. Thereafter, fresh cultures were prepared from maintenance cultures.

Bacteria were inoculated into 10 ml liquid medium to prepare a starter culture prior to dispensing a 1% inoculum into larger volumes for further growth. All liquid cultures were incubated in an orbital incubator (Gallenkamp, Widnes, Lancs.) at 120-150 rpm. Cultures were grown to early stationary phase and purity determined by Gram-staining.

2.2 MEASUREMENT OF BACTERIAL CONCENTRATIONS

Total counts Whole cell counts performed in a haemocytometer (Thoma, Hawksley, England).

Viable counts Serial ten-fold dilutions of bacteria were prepared in sterile phosphate-buffered saline (PBS) (50 mM phosphate buffer, pH7.4, containing 0.15 M NaCl) and volumes of 100 μl were plated on nutrient agar plates. Plates were incubated at 37°C for up to 24 h and numbers of colonies established using a colony counter (Gallenkamp, Loughborough, Leics.).

Measurement by absorbance Measurements of absorbance (A) were made at 525 nm (spectrophotometer, Unicam, Cambridge, England). PBS was used as a blank and as a diluent for absorbance measurements. A standard

curve of log bacterial numbers (as determined by viable counts) against A_{525} was constructed. An absorbance of 0.5 at 525 nm represented approximately $1x10^8$ cells ml^{-1}.

2.3 MINIMUM INHIBITORY CONCENTRATIONS

The minimum inhibitory concentration (MIC) of each antibiotic was determined for each strain by the agar dilution method on iso-sensitest agar in twofold dilutions. A multipoint inoculator (Mast Laboratories Ltd., Liverpool, England) delivered a standard inoculum from bacterial suspensions adjusted spectrophotomically, containing approximately $1x10^4$ cells ml^{-1}.

2.4 ABSORPTION OF SERUM WITH WHOLE CELLS

Heat-inactivated sheep serum was subjected to a series of absorptions with either identical or unrelated *E. coli* whole cells to those being cultured in the absorbed serum. Cultures of washed bacteria were resuspended to a density of $1x10^9$ cells ml^{-1}. Bacterial suspensions (1 ml) were harvested by centrifugation at 3,000 g for 5 min. After removal of supernatant, bacteria were resuspended in 10 ml of serum, incubated for 15 min at room temperature, and recentrifuged. The supernatant was then added to another pellet of cells and the process repeated. This step was repeated at least three times for each absorbing cell type to ensure maximal absorption of antibodies by bacteria. Samples of absorbed and unabsorbed serum were compared in growth study experiments.

2.5 PREPARATION OF SMOOTH LIPOPOLYSACCHARIDES

Extraction of LPS from organisms expressing the S-LPS phenotype was based on the aqueous phenol method of Westphal & Lüderitz (1954).

Eight, 2 litre flasks containing 1 litre of medium were inoculated with a 1% volume of overnight starter culture grown in the equivalent medium. The flasks were incubated in an orbital incubator (120 rpm) at 37°C overnight. Cells were harvested by centrifugation at 15,000 g using a KSB continuous flow system (Dupont UK Ltd., Stevenage, Herts.), before washing twice in PBS with centrifugation (Sorvall RC-5B refrigerated superspeed centrifuge, Dupont) at 10,000 g for 10 min. The bacterial pellet was frozen at -20°C, lyophilized and weighed.

A finely divided lyophilized bacterial mass was resuspended to a concentration of 5% w/v in distilled water and heated to 67°C in a water bath. Meanwhile, 90% w/v aqueous phenol was prepared by dissolving 90 g of phenol (BDH) in 10 ml of distilled water at 45°C, before making the volume up to 100 ml with distilled water. A volume of phenol equal to that of the bacterial suspension was heated to 67°C in a water bath. The prewarmed bacterial suspension and phenol solution were mixed and stirred at 67°C for 15 min. The mixture was transferred to 50 ml centrifuge tubes and cooled in ice to allow separation of the phenol and aqueous phases. The tubes were then centrifuged at 10,000 g for 15 min to complete separation of the phases. The upper aqueous phase containing the LPS was carefully removed and the extraction procedure repeated on the lower phenol phase. The pooled aqueous phases were transferred to dialysis tubing (Medical International Ltd., London) (washed and boiled for 10 min in distilled water) and dialysed against running tapwater overnight, until the smell of phenol was no longer detectable. The solution was centrifuged for 15 min at 10,000 g to remove any insoluble deposit. The dialysed extract was then concentrated by rotary evaporation (Buchi Rotavapor-RE111, Switzerland) to approximately one-fifth of its

original volume. This was proceeded by ultracentrifugation (Sorvall ultracentrifuge - OTD65B, Dupont) of the solution at 100,000 g for 3 h. The resultant gelatinous pellet was resuspended in distilled water with the aid of a syringe fitted with a 23-gauge needle and recentrifuged. The final pellet was suspended in a small volume of pyrogen-free water, lyophilized and weighed. The LPS was stored at -20°C until required.

2.6 PREPARATION OF ROUGH LIPOPOLYSACCHARIDES

Bacteria were cultured and lyophilized as described for S-LPS preparations. The aqueous phenol, chloroform, petroleum ether (PCP) method of Galanos *et al* (1969) incorporating the diethyl ether precipitation of LPS described by Qureshi *et al* (1982), was used to prepare LPS expressing the R-LPS phenotype.

The extraction solvent (PCP) consisted of 90% phenol (Section 2.5), chloroform and petroleum spirit (40°-60°C boiling point) in the proportions 2:5:8 by volume. Lyophilized bacteria were resuspended in PCP at approximately 25% w/v, stirred for 2 min below 20°C, followed by centrifugation at 10,000 g for 15 min. The supernate was filtered through Whatman No 1 filter paper into a round bottomed flask. The centrifuged pellet was re-extracted by the same procedure, and the filtered supernates pooled. Chloroform and petroleum were removed from the supernate by rotary evaporation.

LPS was precipitated by the addition of six volumes of diethyl ether/acetone (1:5 by volume) to one volume of phenol solution. After standing for 1 h the LPS was sedimented by centrifugation at 5,000 g for 10 min. The pellet was washed three times in diethyl ether/acetone. The final pellet was dried under vacuum until the smell

of ether/acetone was no longer detectable, and resuspended in 5 ml of distilled water with the aid of a syringe fitted with a 23-gauge needle. LPS was recovered by ultracentrifugation at 100,000 g for 4 h. Finally, the LPS was taken up in a minimum amount of pyrogen-free water and stored at -20°C until used.

2.7 DEIONIZATION OF LIPOPOLYSACCHARIDE BY ELECTRODIALYSIS

Deionization of LPS was based on the method described by Galanos & Lüderitz (1975) using apparatus developed by ISCO, Nebraska, USA. LPS was suspended in pyrogen-free water (5-10 mg ml^{-1}) and placed in a three-chambered electro-dialysis cell, before adding distilled water to the two electrode chambers. A voltage of up to 500 V was maintained across the cell. The contents of the chambers was replaced several times over 3-4 h when the pH in the cathodic chamber rose. The precipitated acid form of the LPS (deionized) was recovered from the anodic side of the cell and suspended in pyrogen-free water. Deionized LPS was solubilized by neutralization with sodium hydroxide.

2.8 PROTEINASE K DIGESTION OF BACTERIA FOR THE PREPARATION OF LIPOPOLYSACCHARIDES

The proteinase K digestion of bacteria with both R- and S-LPS types (Hitchcock & Brown, 1983) was used to prepare LPS for analysis by PAGE and immunoblotting. Bacterial cultures were harvested by centrifugation and washed twice in PBS. The density of washed bacteria was adjusted to an A_{525} of between 0.5 and 0.6 as measured spectrophotomically. Bacterial suspension (1-5 ml) was transferred to an Eppendorf tube (Elkay) and the bacteria sedimented by microcentrifugation at 10,000 g for 3 min. The pellet was resuspended in 50 μl of single strength PAGE sample buffer (see Section 2.10) and heated

at 100°C for 10 min. On cooling, 10 μl of sample buffer containing 25 μg of proteinase K (Sigma protease Type X1) (2.5 mg ml^{-1} in sample buffer) was added followed by incubation in a 60°C water bath for 60 min. Samples were stored at -20°C.

2.9 PREPARATION OF OUTER MEMBRANE PROTEINS

Bacterial cultures were harvested by centrifugation at 10,000 g for 10 min. Cells were washed twice in PBS and centrifuged as above. The bacterial pellet was resuspended in 13.5 ml distilled water followed by the addition of Sarkosyl (7% w/v solution of sodium N-lauroyl sarcosinate (Sigma)) at 0.7% w/v. Samples were sonicated (Microson, Ultrasonic Cell Disruptor, Heat Systems-Ultrasonics Inc., NY, USA) for six 1 min bursts with intervals of 30 s at an amplitude of 6-10 μm on iced water. Whole cells were removed by centrifugation at 6,000 g for 10 min. The suspension was centrifuged at 50,000 g for 1 h and insoluble outer membrane was harvested and washed once in 5 ml of distilled water by centrifugation at 50,000 g at 4°C for 1 h. The pellet was resuspended in 0.5 ml of distilled water by repeated aspiration into a syringe through a 26-gauge needle. The suspension was stored at -20°C.

2.10 PREPARATION OF SAMPLES FOR POLYACRYLAMIDE GEL ELECTROPHORESIS

Sample buffer (pH6.8) contained 0.0625 M Tris (BDH) at pH6.8 in which 2.0% w/v SDS (BDH Specially Pure), 10% v/v glycerol (BDH), 1% v/v 2-mercaptoethanol (BDH) and 0.001% bromophenol blue (BDH) were present. Double strength sample buffer (pH6.8) was made as above with concentrations of all constituents doubled.

Lipopolysaccharides Proteinase K digests were prepared as described

above and samples were loaded onto the gels at 10 μl per track for silver staining, or 20 μl for immunoblotting.

Protein samples The protein concentration of OMPs and purified MAbs were determined by the method of Lowry *et al* (1951). Samples containing 25 μg protein were mixed with an equal volume of double strength sample buffer and were denatured by heating in a water bath at 100°C for 2 min.

2.11 POLYACRYLAMIDE GEL ELECTROPHORESIS

PAGE was performed on acrylamide slab gels with the buffer system of Laemmli (1970). PAGE of proteinase K samples for subsequent silver staining and immunoblotting was carried out with SDS-free gel buffers (Pyle & Schill, 1985). The following buffers and solutions were used in PAGE:

a) Separating gel buffer (double strength, pH8.8) consisted of 0.75 M Tris-HCl at pH8.8 to which 0.2% w/v SDS was added. (SDS omitted for SDS-free buffer).

b) Stacking gel buffer (double strength, pH6.8) consisted of 0.25 M Tris-HCl at pH6.8 to which 0.2% w/v SDS was added (SDS omitted for SDS-free buffer).

c) Acrylamide stock solutions (40% w/v) contained 100 g acrylamide (BDH Electrophoresis Grade) and 2.7 g methylene bis acrylamide (BDH Electrophoresis Grade) made up to 250 ml with distilled water.

d) Electrode buffer (pH8.3) consisted of 0.025 M Tris (BDH), 0.192 M glycine (BDH Chromatographically Homogenous) and 0.1% w/v SDS (BDH).

Non-gradient gels

The separating gel was prepared as described in Table 4, with

Table 4. Preparation of polyacrylamide gels.

Reagent	Volume (ml) to give acrylamide concentration of:				
	5%	12%	14% Separating gel	20%	4% Stacking gel
Distilled water	4.7	5.2	3.45	1.3	3.5
Separating buffer	6.6	17.5	17.5	8.6	-
Stacking buffer	-	-	-	-	5.0
Acrylamide solution (40% w/v)	1.6	10.5	12.25	-	1.0
Acrylamide solution (50% w/v)	-	-	-	6.9	-
TEMED	0.01	0.05	0.05	0.012	0.02
Ammonium persulphate (15mg/ml)	0.32	1.75	1.75	0.41	0.5

deaeration under vacuum prior to addition of TEMED (NNN'N'-tetramethyl-1,2-diamino ethane (BDH Electran) and ammonium persulphate (BDH). The gel solution was poured between glass plates (160 mm x 125 mm x 1.5 mm) previously wiped clean with methanol and sealed with molten vaseline. The gel was overlayed with water saturated butan-2-ol (BDH) and allowed to set. After removal of butan-2-ol, the stacking gel (prepared in a similar manner) (Table 4), was poured onto the separating gel. A comb was inserted and the gel allowed to set. On removing the comb, the gel was secured within an electrophoresis tank (hand built by Mr J Duffus, Dept of Medical Microbiology, University Medical School, Edinburgh) and electrode buffer added.

Gradient gels

Prepared as described above, except for the separating gel which was composed of a gradient of between 5% and 20% from top to bottom. The gradient was established using a gradient mixer.

Samples, prepared as described above, were loaded into the wells of the stacking gel. These were electrophoresed through the stacking get at a constant 60 V and through the separating gel at a constant 150 V until the dye front had run 8 cm. Electrophoresis was proceeded by staining or immunoblotting.

2.12 SILVER STAINING OF POLYACRYLAMIDE GELS FOR LIPOPOLYSACCHARIDE

The LPS separating gels were stained with silver by the method of Tsai & Frasch (1982), modified by Hancock and Poxton (1988). The following reagents and solutions were used in the silver staining procedure:

a) Fixative consisted of 7% w/v acetic acid (BDH General Purpose) and

25% propan-2-ol (BDH General Purpose).

b) Oxidizing solution contained 1.05 g periodic acid (BDH General Purpose) in 150 ml of distilled water to which 4 ml of fixative was added.

c) Ammoniacal silver nitrate solution was made up by mixing 1.4 ml ammonia solution (BDH General Purpose) with 21 ml of 0.36% w/v sodium hydroxide (BDH) and the slow addition of 4 ml silver nitrate (BDH) accompanied by vigorous agitation. The solution was made up to 100 ml with distilled water.

d) Developing solution consisted of 0.019% v/v formaldehyde (BDH) solution containing 0.005% w/v citric acid (BDH).

The gel was placed in fixative overnight followed by oxidation in freshly prepared periodic acid for 5 min. After frequent washing over a 4 h period in distilled water, fresh ammoniacal silver nitrate solution was added for 15 min. Following at least four washes in distilled water over 40 min, gels were transferred to freshly made developing solution. On development the gel was washed repeatedly in large volumes of distilled water. The gel was stored in the dark. (All reaction steps were shaken following fixation).

2.13 COOMASSIE BLUE STAINING OF POLYACRYLAMIDE GELS FOR PROTEIN

The Coomassie blue stain described by Hancock & Poxton (1988) was used. Solutions were made up in distilled water and included:

a) Solution 1 - 2% v/v propan-2-ol (BDH General Purpose), 10% v/v acetic acid (BDH General Purpose) and 0.05% w/v Coomassie brilliant blue R-250 (BDH).

b) Solution 2 - 10% v/v propan-2-ol, 10% v/v acetic acid and 0.005% w/v Coomassie blue.

c) Solution 3 - 10% v/v acetic acid and 0.0025% w/v Coomassie blue.

d) Solution 4 - 40% v/v methanol (BDH General Purpose) and 10% v/v acetic acid.

e) Solution 5 - 10% v/v acetic acid.

The gel was placed in Solution 1 overnight and then sequentially through Solutions 2-5, each for 30-60 min at room temperature with gentle shaking throughout.

2.14 IMMUNOBLOTTING

This was based on the method of Towbin *et al* (1979) as described by Hancock & Poxton (1988). LPS components separated in PAGE were transferred to nitrocellulose (NIC) paper (pore size 0.2 μm) (Schleicher & Schuell, Dassel, Germany) for immunochemical analysis. The following reagents and solutions were used:

a) Electrode transfer buffer (pH8.3) - contained 12 g TRIS (BDH General Purpose), 57.68 g glycine (BDH Chromatographically Homogeneous) in 4 litres distilled water and 1 litre methanol (BDH General Purpose).

b) Tris buffered saline (pH7.5) (TBS) - contained 4.84 g Tris (BDH), 58.48 g NaCl (BDH) and 2 litres distilled water.

c) Tween-Tris-buffered saline (pH7.5) (TTBS) - as for TBS, containing 0.025% v/v Tween 20 (Sigma).

d) Blocking solution - as for TBS containing 3% w/v gelatin (Bio-Rad EIA Purity Grade, Bio-Rad Laboratories Ltd., Watford, Herts.).

e) Antibody/conjugate diluent - as for TBS containing 1% w/v gelatin (Bio-Rad).

f) Horseradish peroxidase (HRP) colour solution - contained 30 mg HRP colour reagent (Bio-Rad EIA Purity Grade) dissolved in 10 ml

methanol (BDH), to which 50 ml TBS containing 60 µl hydrogen peroxide (30% w/v BDH General Purpose) was added.

Electrophoretic transfer of antigens from polyacrylamide gels to nitrocellulose

The gel was removed from the PAGE apparatus and placed in the Scotchbite™ pad cassette of the blotting apparatus (hand made by Mr J Duffus, Dept. Medical Microbiology, University Medical School, Edinburgh). The gel was covered with a sheet of NIC presoaked in transfer buffer before closing the cassette, sandwiching the gel and NIC paper between the two pads. The assembled cassette was then placed into the immunoblotting tank containing transfer buffer, ensuring the gel was placed towards the cathode and the NIC towards the anode. A constant current of 40 mA was applied overnight at 4°C.

Immunological staining of antigens

After transfer, the NIC was removed and washed in TBS for 10 min before placing in blocking solution for 30-45 min. The blocked NIC was transferred into antibody buffer containing a 1:10 dilution of MAb (shown to give optimum staining). The NIC was rinsed briefly in distilled water and washed for two 10 min periods in TTBS, followed by incubating with a HRP-conjugated secondary antibody (diluted 1:500 in antibody diluent) for 1 h. Conjugates used included an anti-mouse IgG (ICN Biomedicals, High Wycombe, Bucks.) and an anti-mouse IgG/M/A (Zymed Laboratories Inc., San Francisco, California, USA). The NIC was rinsed in distilled water and washed in TTBS as above. Binding of antibody to separated antigenic determinants was visualized by addition of freshly prepared HRP colour development reagent to the NIC. The reaction was stopped when appropriate by washing the membrane in

several changes of distilled water. The blot was dried and stored in the dark. (After the blocking step, gentle shaking was used throughout).

2.15 IMMUNOELECTRON MICROSCOPY

Immunoelectron microscopy was performed by a modification of the method described by Hancock & Poxton (1988). Bacterial cultures were harvested and washed twice in 0.01 M sodium cacodylate buffer, pH7.2. Cells were fixed in cacodylate buffer containing 2% w/v paraformaldehyde (prepared by heating the 2% solution to 60°C, allowing it to cool, adding 1 M NaOH dropwise until clearing occurs, and then adjusting to pH7.2 by addition of 1 M HCl) and 0.1% v/v glutaraldehyde for 1 h at 4°C. After washing in cacodylate buffer, cells were dehydrated twice in graded alcohols and finally rinsed twice in 100% ethanol that had been dried by filtering through sodium sulphate. Samples were embedded in London Resin white and dried at 60°C for 18 h. Thin sections were cut in an ultramicrotome, and placed on nickel grids.

The immunoassay was carried out at room temperature by placing sequentially in:

a) 1% w/v bovine serum albumin (BSA) in PBS, pH7.2, for 15 min to act as a blocking agent.
b) Antibody solution containing a 1:10 dilution of MAb in antibody diluent (see ELISA methodology Section 2.19) for 2 h.
c) 0.1% BSA w/v in 20 mM TBS, pH8.2, to act as a holding buffer prior to washing under a succession of drops of approximately 8 ml TBS from a burette.
d) 1% w/v BSA-TBS for 15 min to act as a blocking buffer.
e) 1:20 dilution 15 nm gold conjugated anti-mouse IgG/IgM (Janssen

Life Sciences, Olen, Belgium) for 1 h.

f) 0.1% w/v BSA-TBS holding buffer prior to washing in the same buffer, followed by distilled water as above.

Immunogold thin sections were viewed with a Hitachi 12A electron microscope at 75 KV.

2.16 WHOLE CELL ELECTRON MICROSCOPY

Simple negative staining was performed using the method described by Hancock & Poxton (1988). Bacteria were harvested from broth, washed once in PBS and resuspended to a milky turbidity in 1% w/v ammonium acetate. Equal volumes of suspension and 2% v/v phosphotungstic acid (neutralized to pH7 with sodium hydroxide) were mixed on the surface of a glass slide. Formvar-coated 400 mesh copper grids were floated on a drop of the specimen for 30 s before removing excess fluid by lightly blotting with filter paper (Whatman No 1). Grids were placed in a desiccator for several min before viewing with the electron microscope. Grids were examined for capsule presence and relative thickness.

2.17 FLOW CYTOMETRY

Washed cultures of bacteria were resuspended to an A_{525} of between 0.5 and 0.6, equivalent to approximately $1x10^8$ cells ml^{-1}. Suspensions (1 ml) were microcentrifuged at 10,000 g for 2 min and the pellets resuspended in 1 ml of MAb culture supernates, diluted 1:10 in dilution buffer (see ELISA methodology, Section 2.19), and incubated for 60 min at 37°C. Samples were washed twice in PBS and 0.5 ml sheep FITC-conjugated anti-mouse IgG or IgM (ICN Biomedicals) (diluted 1:100 in dilution buffer) was added, and incubated for 60 min at 37°C. Following further washings in PBS, the pellet was resuspended in 1 ml

PBS containing 0.5% formaldehyde.

Prepared samples were diluted 1:50 in PBS, and the fluorescence of stained bacteria quantified using an EPICS 'C' (Coulter Electronics) flow cytometer equipped with a 5 watt argon ion laser, operating at 500 mW and exciting at 488 nm. Cells were passed through the beam at approximately 500 s^{-1} from a standard 76 μm flow cell tip. Background noise and clumps of cells were excluded by a gate on the log forward angle light scatter. Cells stained with FITC conjugate, but no primary antibody, provided a background staining level, set at 1% ± 0.5% by adjusting the voltage applied to the green fluorescence log (GFL) photomultiplier tube. A total of 50,000 cells were analysed from each sample and the percentage of cells exhibiting positive staining GFL was calculated by the EPICS 'Stat Pack' programme.

Cell sorting

Viable bacterial cells were sorted in the flow cytometer on the basis of differential MAb binding (fluorescence). The instrument was sterilised with 50% ethanol. Bacteria were prepared as previously described but omitting the fixation step. The light scatter gain was set at maximum, a horizontal gate set to exclude debris, and vertical fluorescence gates set to separate cell populations of interest. Sorted bacteria were collected in nutrient broth and subcultured on nutrient agar medium. Single clones were picked, expanded, and reanalysed by flow cytometry.

2.18 *IN VIVO* ANIMAL MODELS

All animal manipulations were performed by Mr J Verth and colleagues (Animal House, University Medical School, Edinburgh) or Dr I R Poxton

(Dept of Medical Microbiology, University Medical School, Edinburgh).

Chamber Implantation

Chambers were constructed and implanted in the mouse peritoneal cavity based on the method described by Patrick *et al* (1984). Chambers were made from 1 cm lengths of 1 ml polypropylene syringe barrels. Two holes were made towards each end of the chamber with a red-hot 25-gauge needle. After autoclaving (15 psi for 15 min), the ends of the chambers were sealed with autoclaved (10 psi for 10 min) 0.22 μm membrane filters (Millipore, Bedford, M.A. USA) using UHU glue (Beecham UHU, Brentford, Middlesex). The *E. coli* culture used to inoculate the chambers was grown overnight in nutrient broth, harvested, washed and diluted in physiological saline to give a chamber inoculum of approximately $1x10^5$ cells ml^{-1}. The chambers were filled through the holes with bacterial suspensions. The two holes were sealed with UHU glue and the chambers transferred to bijou bottles containing saline. The C57B16 male mice were anaesthetized by intraperitoneal injection of Avertin (prepared by dissolving 0.315 g tribromoethyl alcohol in 0.25 ml isoamyl alcohol in a water bath at 37°C followed by the addition of 10 ml physiological saline) at 0.2 ml/10 g body weight. Two chambers were implanted into each animal through a small longitudinal incision in the abdomen. The chambers were removed after the required incubation period (see Results) before harvesting the cells by centrifugation and washing twice in PBS. The numbers of viable bacteria in each chamber were determined. Samples were finally prepared for analysis using techniques described elsewhere in this section.

The mucin-haematin septicaemia model

The infection model was a modification of the method described by

Appelmelk *et al* (1986b). Mucin and haematin reagents were prepared as follows:

a) Mucin, 24% w/v was prepared by suspending 2.4 g mucin (porcine stomach mucin, type II,, Sigma) in 7 ml PBS adjusting to pH7.4 with 1 M NaOH. Volume was made up to 10 ml with PBS, and material sterilised by autoclaving (10 psi for 10 min).

b) Haematin HCl, 0.234% w/v, was prepared by dissolving 23.4 mg haematin HCl (BDH) in 2 ml 0.05 M NaOH. Approximately 6 ml PBS was added, adjusting to pH7.4 with 1 M HCl. The volume was made up to 10 ml with PBS. The solution was filter sterilised through 0.2 μm pore size membrane filters (Millipore).

Equal volumes of the mucin and haematin preparations were mixed and stored at 4°C. Overnight cultures of *E. coli* were harvested by centrifugation and washed twice in PBS. Bacterial concentration was adjusted to a total cell count of approximately 200 cells ml^{-1}. The mucin-haematin suspension was diluted with an equal volume of cell suspension, and 0.5 ml inoculated into the peritoneum of C57B16 male mice. Retrospective viable cell counts of inocula samples were determined.

Mice were bled via the vena cava up to 24 h after inoculation, and the blood transferred to sterile universals containing 20 units of heparin. Bacteria were recovered from blood components using Sepracell-MN (Sepratech Corporation, Oklahoma City, USA). Equal volumes of anti-coagulated blood and Sepracell-MN were mixed gently in a centrifuge tube. Samples were centrifuged for 20 min at 1,500 g to sediment red blood cells. The first 20% of the supernatant, containing bacteria and blood mononuclear cells was mixed with four volumes of PBS-BSA (0.1%

w/v) and centrifuged at 300 g for 10 min to remove mononuclear cells. Bacteria were recovered from the supernatant by centrifugation at 3,000 g and prepared for analysis.

2.19 ENZYME LINKED IMMUNOSORBENT ASSAY

Polystyrene microplate 8-well strips (Immuno module Polysorp F8, Nunc, Inter Med, Kampstrip, Roskilde, Denmark) were used in all ELISA experiments. Frame-modules were used to hold strips, forming a 'plate'. Mini-sorb tubes (Nunc) were used for making antigen and antibody dilutions. The following diluents and buffers were used for various ELISA procedures:

a) Coating buffer (pH9.6) consisted of 0.05 M carbonate/bicarbonate buffer (6.2g L^{-1} $Na_2CO_3H_2O$ and 4.2g L^{-1} $NaHCO_3$) and 0.05% w/v sodium azide (Sigma).

b) Post-coat buffer consisted of PBS (pH7.2) containing 5% v/v BSA (ICN Biomedicals) and 0.05% w/v sodium azide.

c) Wash buffer consisted of PBS (pH7.2) containing 0.05% v/v Tween-20 (Sigma) and 0.05% w/v sodium azide.

d) Dilution buffer consisted of PBS (pH7.2) containing 0.05% v/v Tween-20, 4% w/v polyethylene glycol 6,000 (Sigma) and 0.05% w/v sodium azide.

ELISA coating procedures

a) Coating with whole bacteria

ELISA strips were coated with 100 μl per well of washed bacteria, resuspended to a density of $2x10^7$ cells ml^{-1} (measured spectrophotometrically) in coating buffer. Coating was promoted by centrifugation at 630 g for 4 min and leaving overnight at room temperature. Plates were washed four times (Ultrawash 1, Dynatech Laboratories Ltd.,

Billingshurst, Sussex) in wash buffer, before post coating at 100 μl per well overnight at room temperature. After further washing, plates were rinsed in distilled water and stored at -20°C until used.

b) Coating with heat killed or sonicated whole cells

Washed bacteria were resuspended to a density of $2x10^7$ cells ml^{-1} in dilution buffer and either heat killed at 100°C for 15 min or sonicated for six, 1 min bursts with 30 s intervals. Preparations were added at 100 μl per well. Following overnight incubation at room temperature, the procedure was as described for whole cell coating.

c) Coating with LPS-polymyxin complex (Scott & Barclay, 1987)

Stock solutions of LPS and polymyxin were made up in distilled water and were sonicated for 30 s prior to mixing. Complexes were formed by adding polymyxin B sulphate (Sigma) at a concentration of 0.2 mM with LPS at a concentration of 0.1 mM. LPS-polymyxin complexes were sonicated for a further 30 s and then stirred for 30 min. The mixture was transferred to cellulose dialysis tubing with a 2,000 molecular weight cut off (Spectrum Medical Industries Inc., Los Angeles, USA) and dialysed overnight at 4°C against distilled water (containing 0.05% w/v sodium azide) to remove excess, unbound polymyxin. Complexes were stored in mini-sorb tubes at -20°C until required. Molarity of *E. coli* R- and S-LPS was determined from the estimated molecular weights of LPS as determined by Morrison & Jacobs (1976):

LPS TYPE	MOLECULAR WEIGHT
Wild type smooth	15,000
Ra and Rb type	4,500
Rc type	4,150
Rd and Re type	3,100

Microwell strips were coated with complexes at 100 μl per well after

diluting 1:50 in coating buffer. Cocktails of different LPS-polymyxin complexes (see Results) were constituted by mixing equal volumes of the relevant complexes. Incubation, washing and post-coating were carried out as described for LPS whole cell coating.

d) Coating with proteinase K digested cell extracts

Cultures of bacteria were harvested and washed twice in PBS before resuspending to an A_{525} of 1.0 (equivalent to approximately $1x10^9$ cells ml^{-1}) in 0.0625 M Tris-HCl pH6.8. Suspensions were heated at 100°C for 10 min. On cooling, proteinase K (Sigma) (2.5 mg ml^{-1} in Tris-HCl, pH6.8) was added, followed by incubation in a 60°C water bath for 60 min. In preliminary experiments, the addition of several volumes of the proteinase stock solution were investigated. A volume of 10 μl (containing 25 μg of proteinase K) per 1.5 ml cell suspension was found to digest the cells efficiently, whilst minimizing the amount of added enzyme. Following digestion, samples were centrifuged at 10,000 g for 5 min to remove any undigested whole cells or large cell-fragments. Digested extracts were then heated at 100°C for 5 min (found to eliminate any residual proteinase K activity).

LPS containing samples were coated to microplates after complexing with polymyxin B sulphate. Complexes were formed by mixing (30 min at room temperature) equal volumes of presonicated 1 mM polymyxin and proteinase K digested sample. The mixture was dialyzed as above to remove excess, unbound polymyxin. In preliminary experiments, coating microwell strips with LPS-polymyxin complexes, doubly diluted in coating buffer from 1:2 to 1:128 were investigated. A coating dilution of 1:16 was shown to give optimal sensitivity, yielding maximal difference in optical density (OD) between coated wells and background

controls. Following overnight coating at room temperature, the procedure was as described for whole cell coating.

ELISA experimental procedures

a) Standard procedure to quantify anti-LPS MAb binding

MAb supernatant fluids were diluted in dilution buffer and added to coated microplates at 100 µl per well in triplicate. Plates were incubated at 37°C for 90 min before washing four times with wash buffer. Urease-conjugated sheep anti-mouse 1 g (Sera Lab Ltd., Crawley Down, Sussex) was diluted 1:500 in diluent, added at 100 µl per well and plates incubated for a further 90 min at 37 C. Plates were washed four times and rinsed with distilled water before urease substrate (Sera Lab) was added at 100 µl per well. Plates were incubated for 60 min at room temperature and reactions stopped by adding 1% w/v thimerosal in distilled water (20 µl per well). The OD of wells was read at 590 nm using an Anthos reader 2001 (Denley Instruments Ltd., Billingshurst, Sussex). Final results were expressed after subtraction of the OD of negative control wells (coated with BSA post coat only) for each MAb.

b) Suspension ELISA

The ability of selected MAbs to bind to a suspension of intact whole cells in ELISA was investigated. Initial stages of the assay were performed in microtubes. Nutrient broth grown bacterial cultures were harvested and washed twice in PBS with centrifugation at 3,000 g for 5 min. Pellets of approximately $2x10^7$ cells were resuspended in 1 ml of each MAb dilution. Tubes were incubated at 37°C for 90 min with occasional shaking, before centrifugation as above. The supernatant was discarded and bacterial pellets washed twice in ELISA wash buffer

with centrifugation. Urease-conjugated sheep anti-mouse Ig was diluted 1:500, added at 1 ml per tube and incubated for a further 90 min at 37°C. Bacteria were recovered by centrifugation, before washing twice in both wash buffer and distilled water. Samples were added to microtitre plates at 100 µl per well (representing the equivalent cell coating concentration of the standard ELISA coating procedure). Coating was promoted by centrifugation at 630 g for 10 min before removing supernatant from wells. Urease substrate was added at 100 µl per well, and plates incubated for 60 min at room temperature before reading at 590 nm.

c) Serial absorption of MAbs on whole bacteria

MAbs were absorbed serially by bacteria of *S. typhimurium* in ascending order of LPS core size: lipid A, Re, Rc and Ra. Lipid A bacteria were prepared by acid hydrolysis of the *S. typhimurium* Re mutant by incubating at 100°C in 1% v/v acetic acid for 1 h. MAb supernate (1 ml), diluted 1:10 in dilution buffer, was added to a 1.5 ml Eppendorf tube containing sedimented, washed bacteria at $1x10^8$ cells ml^{-1}. Bacteria were resuspended in the MAb solution, incubated for 15 min at room temperature and centrifuged at 10,000 g for 5 min using a micro-centrifuge. The supernatant was then added to another pellet of cells and the process repeated. This step was repeated three times for each absorbing bacterial strain to ensure maximal absorption of antibodies by bacteria. A sample of MAb solution was removed after absorption by each bacterial strain in the serial sequence for ELISA tests. The ELISA procedure was as previously described.

d) Inhibition of ELISA

Anti-LPS MAb, diluted as appropriate in dilution buffer, was added to

an equal volume of each dilution of various inhibitor preparations (as specified in the Results). Samples were preincubated at 37°C for 30 min with occasional shaking before adding to the ELISA. Modifications of this method are as outlined in the Results.

2.20 PURIFICATION OF MONOCLONAL ANTIBODIES

Selected MAbs were purified for use in the LPS detection assay system only.

Concentration of MAbs from bulk growths

Culture supernatant from bulk growth (1 litre) of MAb producing clones was centrifuged at 10,000 g for 10 min to remove cellular debris. The MAb supernates were concentrated 20-fold by a Minitan tangential-flow system (Millipore), employing Minitan pore filters (Millipore) with a molecular weight cut-off of 30,000. The system was purged with 0.1 M sodium hydroxide (BDH), copious amounts of pyrogen-free water and primed with a small quantity of RPMI (Gibco, Paisley, Renfrewshire) between each concentration. Activity of MAbs was checked by ELISA.

Ammonium sulphate precipitation of immunoglobulins

A modification of the method described by Hardy (1986) was used. Immunoglobulin was precipitated by the addition of crystalline ammonium sulphate (Sigma Grade III) to the concentrated solution containing MAb to give 50% saturation (equivalent to a final concentration of 0.313 g ml^{-1}). Thus 3.13 g of ammonium sulphate was slowly added to 10 ml of MAb preparation with stirring, and allowed to dissolve. The precipitate was stirred for 2 h at room temperature before harvesting by centrifugation at 10,000 g for 60 min at 4°C. The pellet was resuspended in 10 ml of 0.1 M $NaHCO_3$ (BDH) before repeating the precipitation

procedure as above. The ammonium sulphate was removed by dialysis for 20 h at 4°C with stirring, against at least three changes of 0.1 M $NaHCO_3$ buffer containing 0.05% w/v sodium azide (Sigma). Activity of the MAbs was checked by ELISA.

Cartridge chromatography

Ammonium sulphate precipitated MAbs were further purified using MemSep™ chromatography cartridges (Millipore). All buffers used for the elution of MAb containing solutions were prepared with Milli-Q® ultra pure water and filtered through Sterivex™-GS 0.22 μm filter units (Millipore).

Protein-A IgG MAbs were purified using a Protein-A MemSep™ 1010 affinity membrane chromatography cartridge. The MAb sample was prepared by diluting 1:2 in sample loading buffer containing 0.05 M Tris-HCl, 0.1 M NaCl, pH8 followed by filtration through a Millex® -GV unit (Millipore). The cartridge was initially equilibrated by passing through ≥45 ml of loading buffer. After loading with the prepared MAb solution using a peristaltic pump, the sample was recirculated several times at a flow rate of 3.6 ml min^{-1}. The cartridge was rinsed with loading buffer until the A_{280} of the effluent was at baseline. MAb was desorbed by passing 0.1 M glycine-HCl, pH2.5 through the system, and A_{280} monitored until it returned to baseline. Eluted MAb was neutralized with 5% v/v 1 M Tris (BDH) before dialyzing against at least three changes of 0.1 M $NaHCO_3$ (BDH) containing 0.05% w/v sodium azide (Sigma). Fractions were checked by ELISA for MAb activity.

DEAE IgM MAbs were purified using a DEAE Mem-Sep™ 1010 ion

exchange membrane chromatography cartridge. MAb samples were diluted 1:2 in sample loading buffer containing 0.02 M Tris-HCl, pH8 followed by filtration through Millex® -GV units (Millipore). Prepared MAb solution was loaded on to the DEAE using a peristaltic pump, and recirculated several times at a flow rate of 3.6 ml min^{-1}. The cartridge was rinsed with loading buffer until the A_{280} of the effluent reached baseline. Protein components were desorbed using a step-wise elution protocol based on increasing the NaCl concentration from 0.1 M to 1 M in 0.05 M increments. Individual fractions were collected and analysed by ELISA for MAb activity. The MAb containing fraction (eluting between 0.15 and 0.2 M NaCl) was dialyzed against at least three changes of 0.1 M $NaHCO_3$ (BDH) containing 0.05% w/v sodium azide (Sigma).

2.21 BIOTINYLATION OF MONOCLONAL ANTIBODIES

Biotinylation of MAbs was based on the method described by Guesdon *et al* (1979). Aminohexanoyl-biotin-N-hydroxysuccinimide ester (AH-BNHS) (Zymed) was used to bind biotin covalently to the MAbs. The protein concentration of purified MAbs was determined by the method of Lowry *et al* (1951). Calculations of quantities of reagents were based on a value of 90 free amino groups per gamma globulin molecule as reported by Habeeb (1966). Various molar ratios (1:1, 2:1, 4:1) of AH-BNHS and free amino groups of IgG and IgM MAb molecules were prepared to determine the optimal binding ratio. To react a 1:1 molar ratio of AH-BNHS with the free amino groups of IgG MAb, 27.5 μl of AH-BNHS (10 mg/ml dissolved in dimethylformamide BDH) was added to 1 ml of MAb (1 mg ml^{-1} in 0.1 M $NaHCO_3$). To react a 1:1 molar ratio of AH-BNHS with the free amino groups of IgM MAb, 22.7 μl of AH-BNHS (10 mg ml^{-1}) was added to 1 ml of MAb (1 mg/ml in 0.1 M $NaHCO_3$). Reactions were performed in

Reacti-vials™ (Pierce Chemical Co., Rockford, Illinois, USA) and were gently stirred for 2 h at room temperature. The reaction mixture was dialyzed for 24 h at 4°C with stirring, against several changes of PBS (pH7.2) containing 0.05% w/v sodium azide (Sigma). After dialysis, an equal volume of glycerol (Sigma) was added and the biotinylated MAbs (bio-MAbs) stored at -20°C. The bio-MAbs were analysed by ELISA.

2.22 STREPTAVIDIN-BIOTIN ELISA SYSTEMS

Prepared bio-MAbs were used in a comparison of a streptavidin-biotin enzyme non-amplification ELISA system, with an amplification system incorporating streptavidin-biotin-enzyme complexes. Buffers, reagents and methodologies used in these ELISA studies were as previously described unless otherwise indicated.

Enzyme-labelled reagents The following enzyme-labelled reagents were used at some stage in the development of the LPS-detection assay: i) streptavidin alkaline phosphatase (SAP) (Zymed) diluted 1:1000 in dilution buffer (Section 2.19); ii) biotinylated alkaline phosphatase-streptavidin (BAPS) complex made up by the addition of 10 μl biotinylated alkaline phosphatase (1 mg ml^{-1}) (Zymed) and 20 μl streptavidin (1 mg ml^{-1}) (Zymed) to 5 ml dilution buffer (PBS, pH7.2, containing 0.05% v/v Tween 20 (Sigma).

Substrate Substrate for the alkaline phosphatase labelled reagents was prepared by dissolving one p-nitrophenyl phosphate tablet (Sigma 104 phosphatase substrate tablets) per 5 ml of substrate dilution buffer, pH9.8 (containing 0.05 M sodium carbonate/bicarbonate, 0.002 M magnesium chloride).

Bio-MAbs were serially diluted (or as appropriate) in diluent and added

to antigen coated microwell strips at 100 μl per well. In some of the investigations (chequerboard titratrions) strips were coated with a series of different antigen concentrations. Plates were incubated for 90 min at 37°C and then washed. Enzyme labelled reagents were added at 100 μl per well and plates incubated for a further 90 min prior to washing. Substrate was added (100 μl per well) and plates incubated for 60 min at room temperature before the OD was read at 405 nm.

2.23 MONOCLONAL ANTIBODY COMPETITION STUDIES

An ELISA competition assay was used to compare the antigen binding sites of different MAbs. Buffers, reagents and methodologies were as previously described. A serial dilution of unlabelled MAb (capable of saturating the binding site at the lowest dilution) was prepared in dilution buffer and added to antigen coated microwell strips at 100 μl per well. Following incubation at 37°C for 90 min, serially diluted bio-MAb (the same as or different from the unlabelled MAb) was added to the unlabelled MAb at 100 μl per well in a chequerboard formation. MAbs were mixed by shaking and incubated for 90 min at 37°C, before washing and adding 100 μl per well alkaline phosphatase conjugated streptavidin (1:1000). Following incubation for a further 90 min at 37°C, plates were washed and substrate added at 100 μl per well. Plates were incubated at room temperature for 60 min before the OD was read at 405 nm.

2.24 CAPTURE ELISA

An LPS capture ELISA, employing different combinations of various MAbs, was developed with the aim of detecting either all *E. coli* core types or specific core and O-serotypes in solution. Solutions, reagents and basic methodologies were as previously described.

The wells of microwell strips were coated with MAb, diluted as appropriate (see Results) in coating buffer. Plates were coated overnight at room temperature before washing, post-coating and storing as previously described. A series of dilutions of purified *E. coli* LPS and heat-killed cells were made in dilution buffer and added at 200 μl per well. Plates were incubated overnight at room temperature. Bio-Mab, diluted as appropriate (see Results) was added at 100 μl per well and plates incubated at 37°C for 90 min before washing. Alkaline phosphatase conjugated streptavidin was added at 100 μl per well and plates incubated for 90 min at 37°C. After further washing, alkaline phosphatase substrate was added at 100 μl per well. The OD of wells was read at 405 nm following incubation for 60 min at room temperature.

The above method was used for detection of LPS in spiked serum samples. Serum was treated in various ways prior to the assay, as described in the Results. Samples were added at 200 μl per MAb coated well. The remainder of the assay was as described above.

RESULTS

CHAPTER 1

CHARACTERIZATION OF ANTI-LIPOPOLYSACCHARIDE MONOCLONAL ANTIBODIES

All MAbs used in this thesis were obtained from fusions carried out in the Dept Surgery, University of Edinburgh, as outlined in the Materials and Methods. The selection of MAbs for use in these studies was based on predetermined reactivity patterns against an extensive panel of both R- and S-LPSs in a LPS-polymyxin extended incubation ELISA (Scott & Barclay, 1987) (see Appendix). Selected MAbs were further characterized in a number of assay systems employing different LPS preparations.

1.1 BINDING OF ANTI-LIPOPOLYSACCHARIDE MONOCLONAL ANTIBODIES AGAINST HEAT-KILLED BACTERIA IN ELISA.

The binding activities of eleven MAb supernates against heat-killed bacteria are shown in Figures 6-16. ELISA antigens included a broad panel of Gram-negative *E. coli* and non-*E. coli* species expressing O-antigen S-LPS and core R-LPS. MAbs 184.2.5.5 (Figure 15) and 185.1.2.2 (Figure 16) reacted exclusively against the *E. coli* serotypes O18 and O6 respectively. Binding of MAb 184.2.5.5 to the rough mutant of *E. coli* O18 reflected the presence of some leaking O-antigen.

MAbs 27.150.3 (Figure 6), 43.11.5.1 (Figure 12) and 43.27.11.2 (Figure 13) were all fully cross-reactive with *E. coli* core types (R1-R4 and K12) and all *E. coli* serotypes. These MAbs showed a weak, if any, response to the R- and S-LPS strains of *S. typhimurium*, whilst only 43.27.11.2 recorded some, although weak reactivity against bacterias *P. aeruginosa* and *K. pneumoniae*. Overall, MAb 27.150.3 produced the strongest reactions against the panel of antigens, whilst MAb

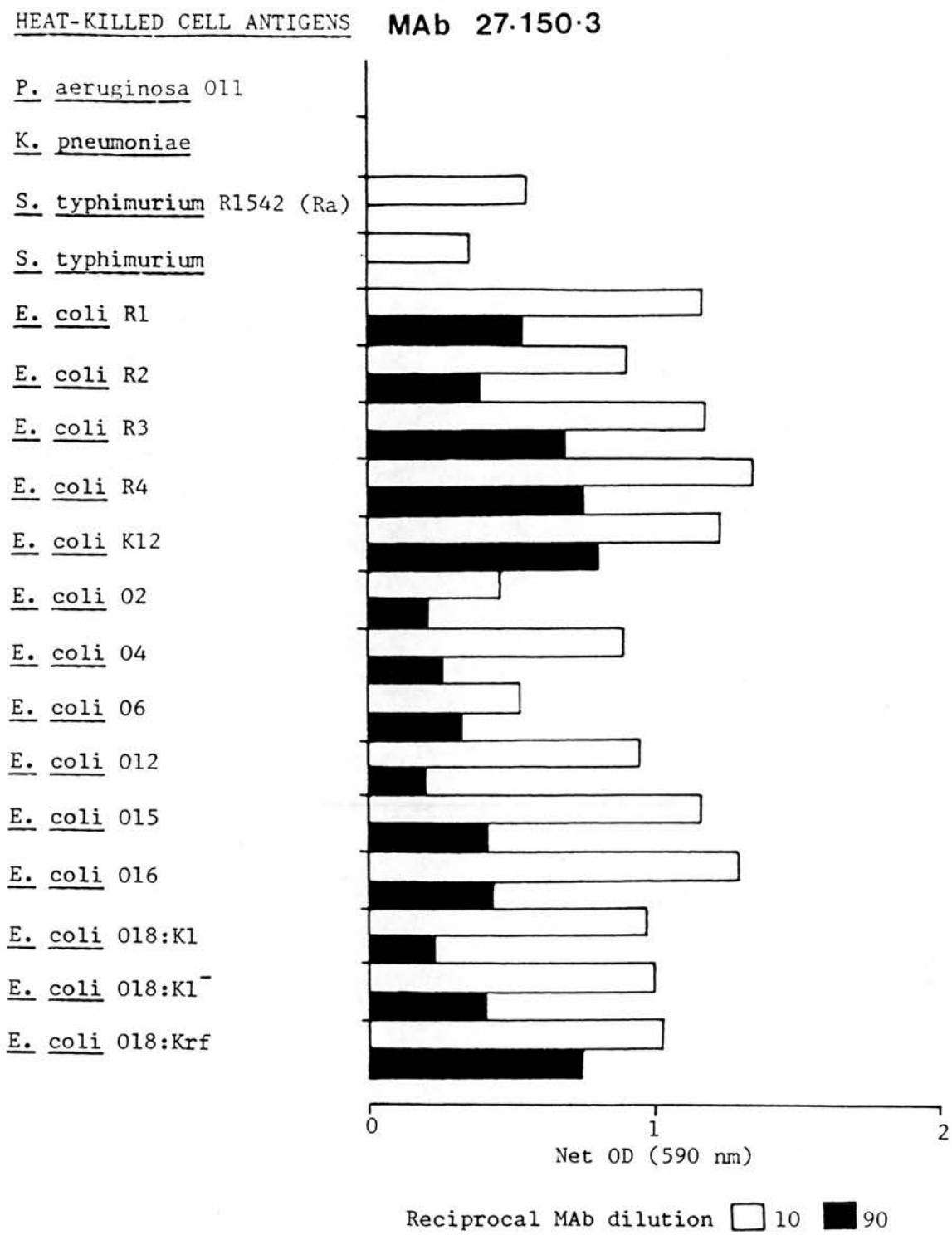


Figure 6. ELISA activity of MAb 27.150.3 against whole, heat-killed *E. coli* and non-*E. coli* rough and smooth cell types.

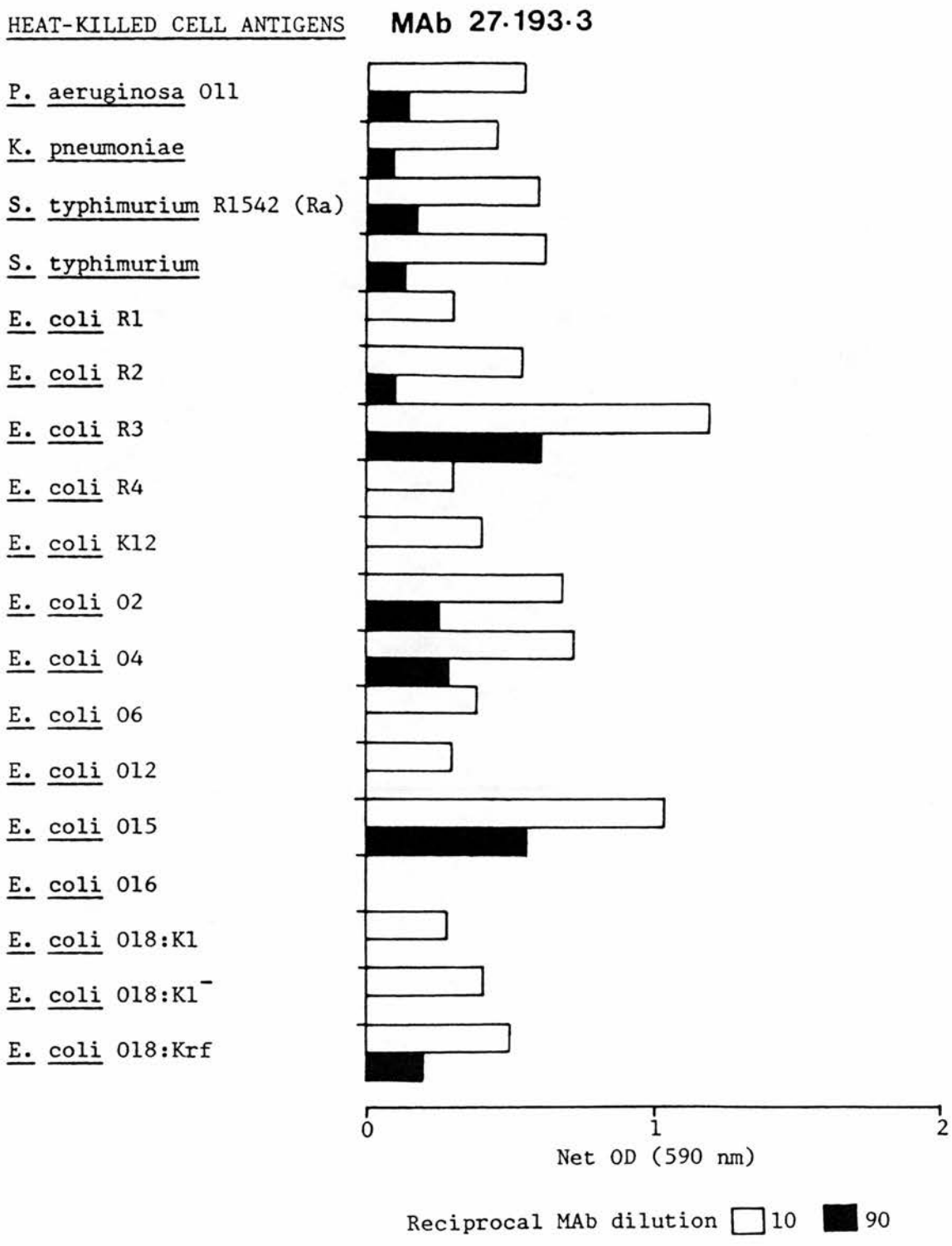


Figure 7. ELISA activity of MAb 27.193.3 against whole, heat-killed *E. coli* and non-*E. coli* rough and smooth cell types.

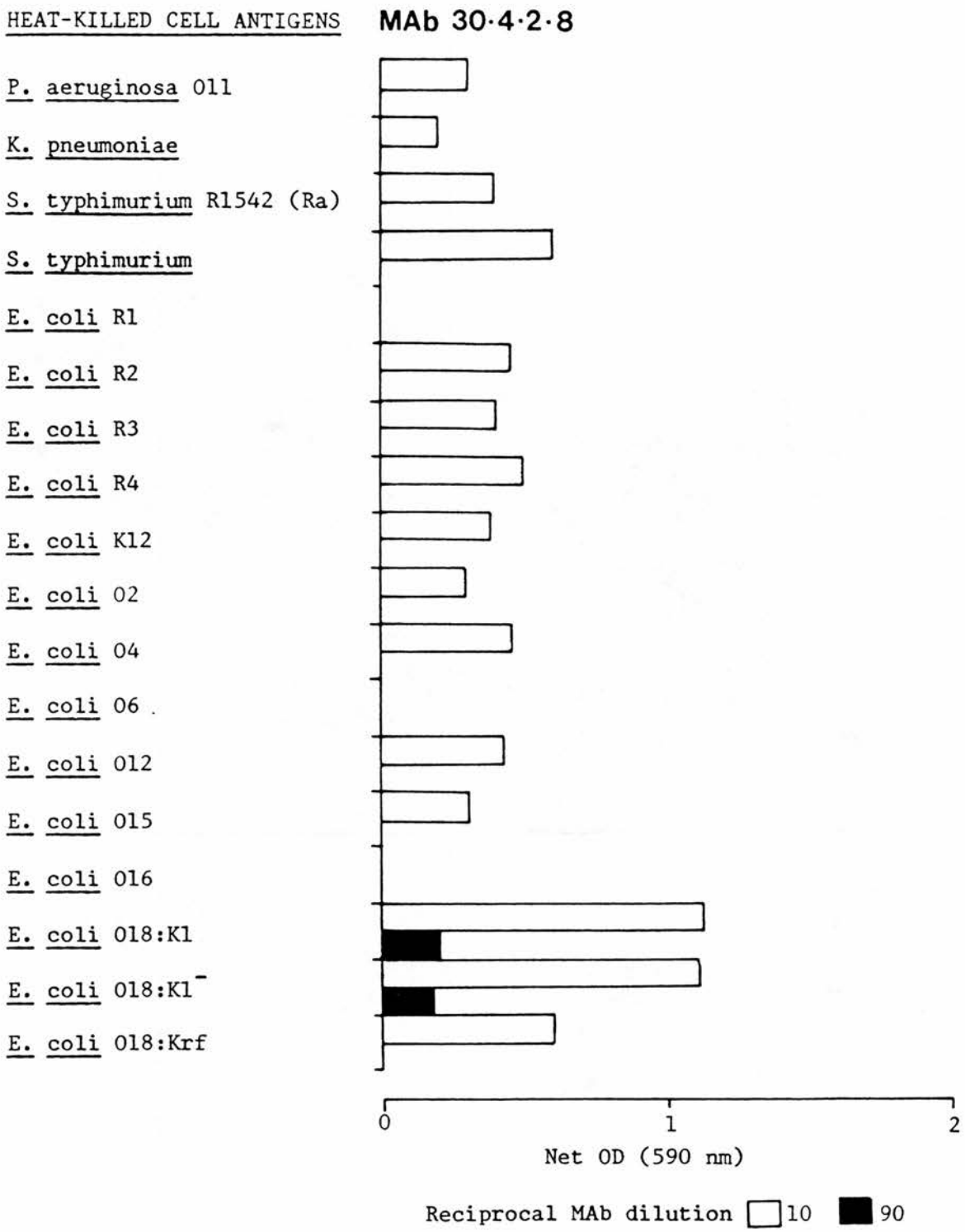


Figure 8. ELISA activity of MAb 30.4.2.8 against whole, heat-killed *E. coli* and non-*E. coli* rough and smooth cell types.

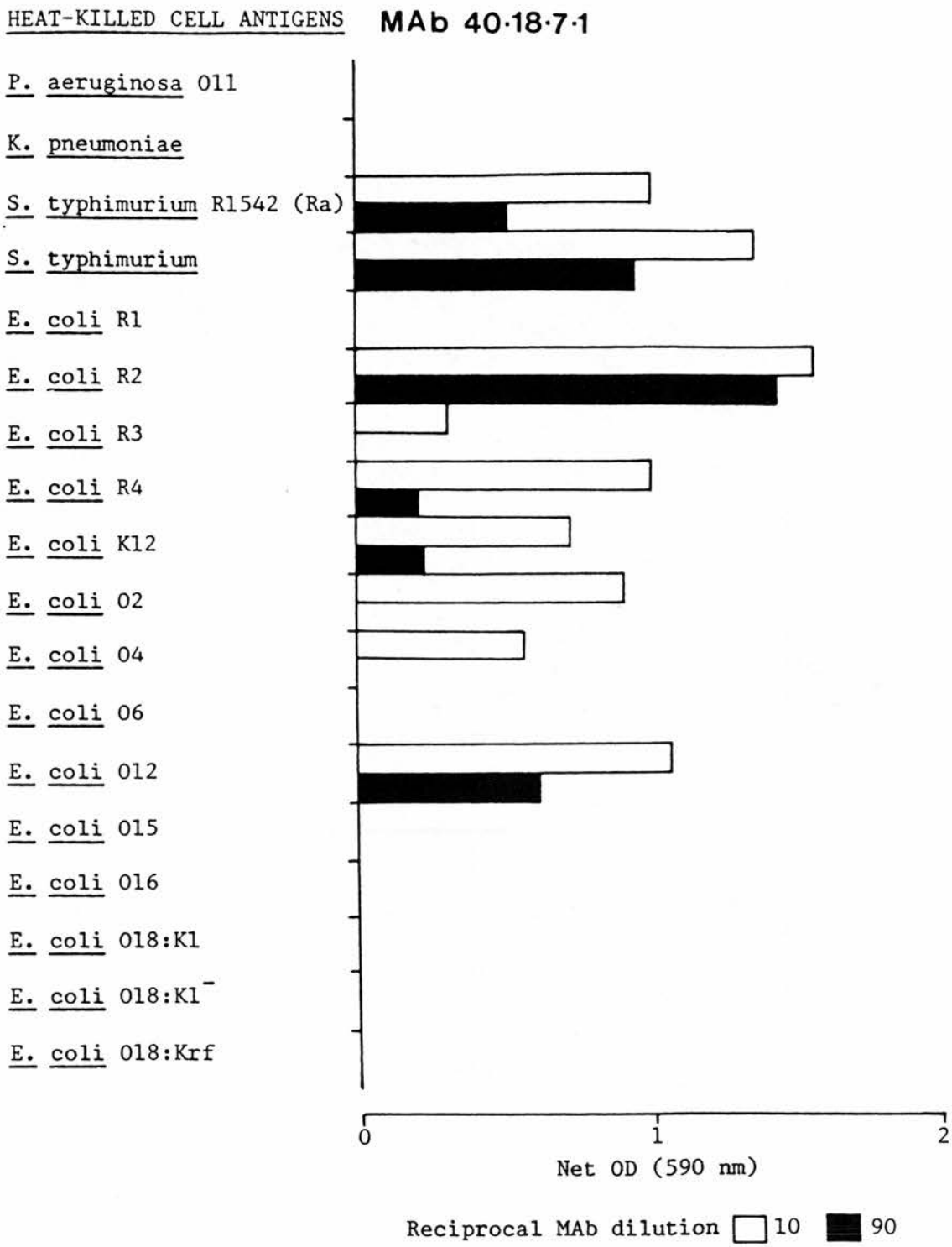


Figure 9. ELISA activity of MAb 40.18.7.1 against whole, heat-killed *E. coli* and non-*E. coli* rough and smooth cell types.

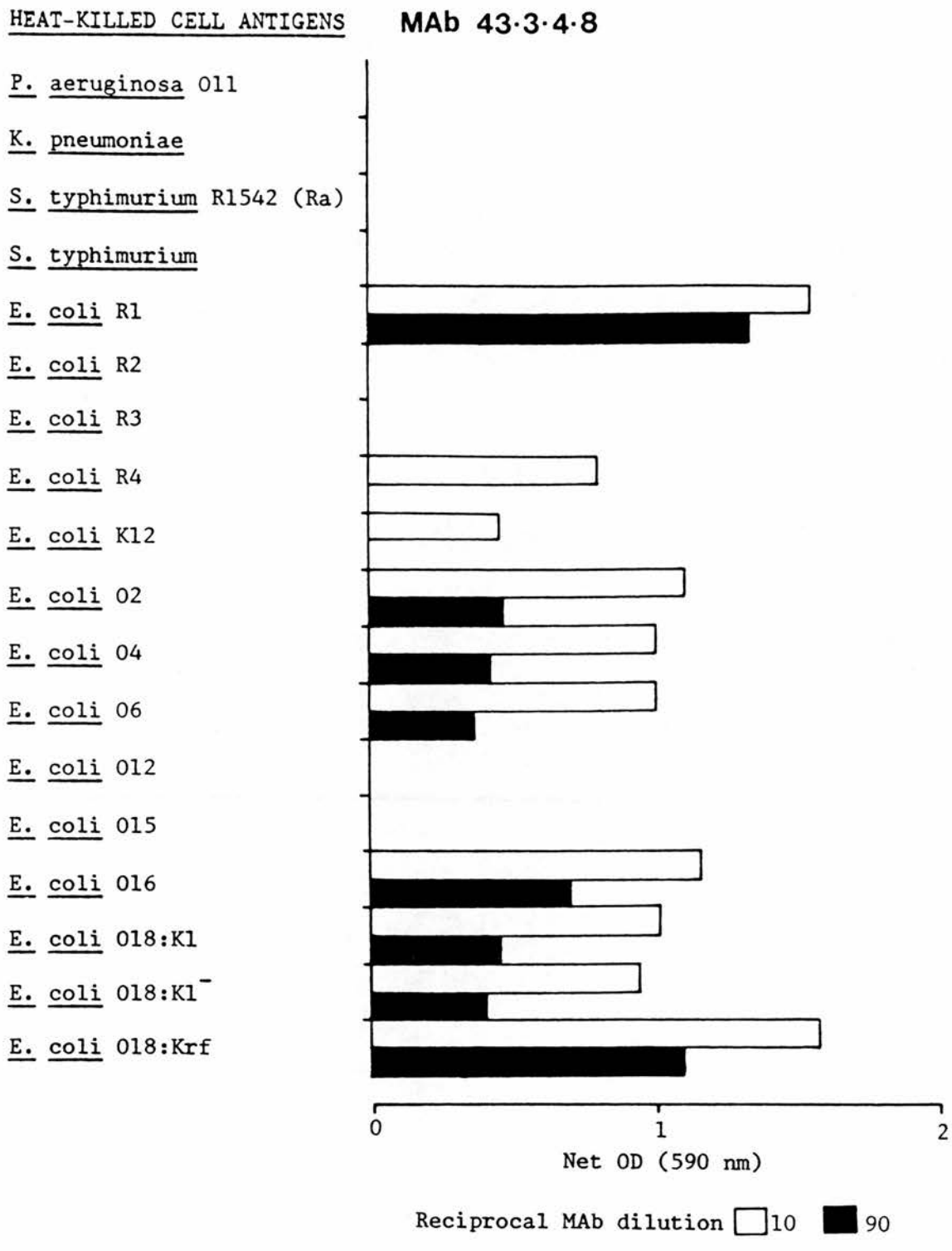


Figure 10. ELISA activity of MAb 43.3.4.8 against whole, heat-killed *E. coli* and non-*E. coli* rough and smooth cell types.

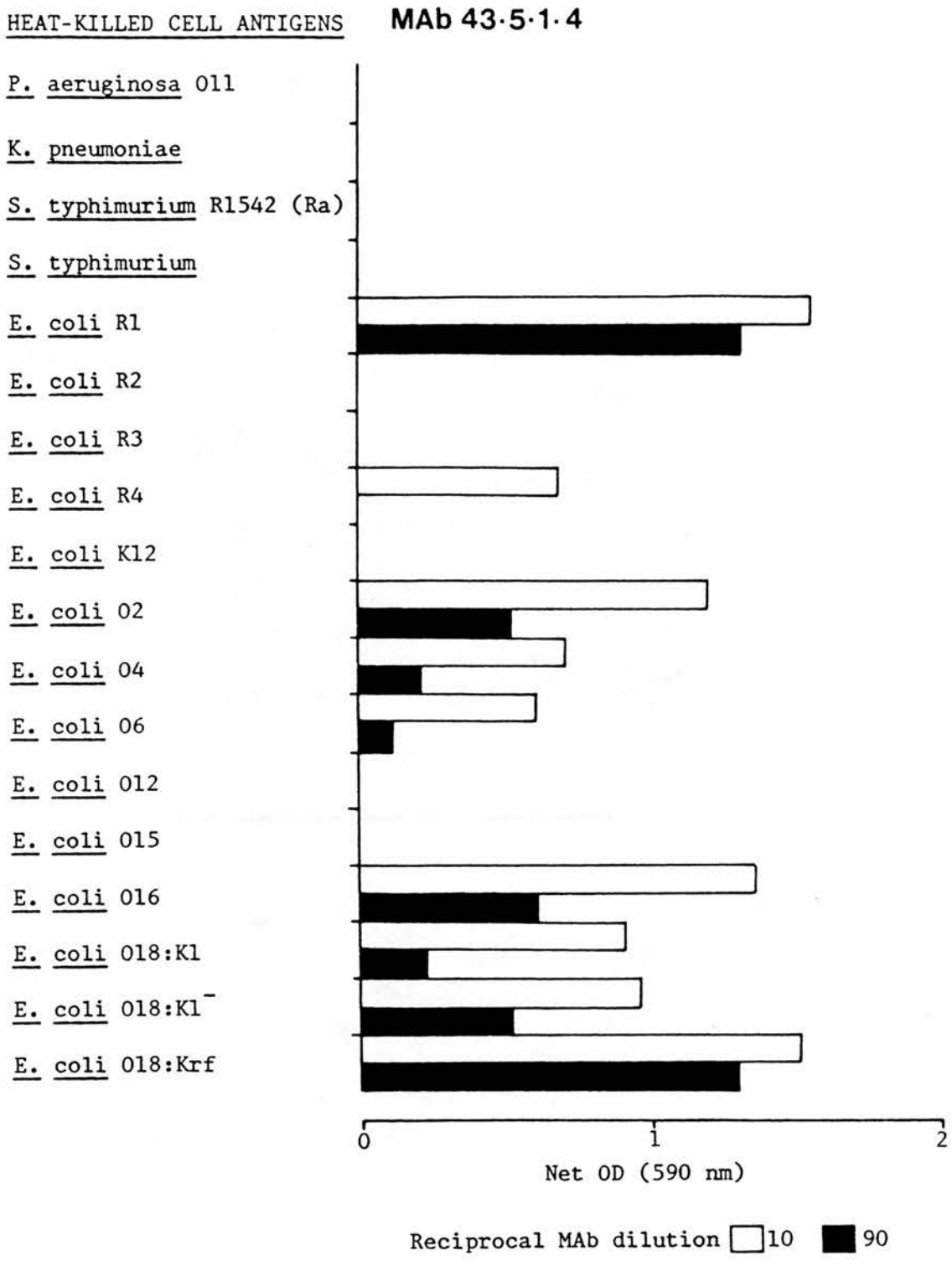


Figure 11. ELISA activity of MAb 43.5.1.4 against whole, heat-killed *E. coli* and non-*E. coli* rough and smooth cell types.

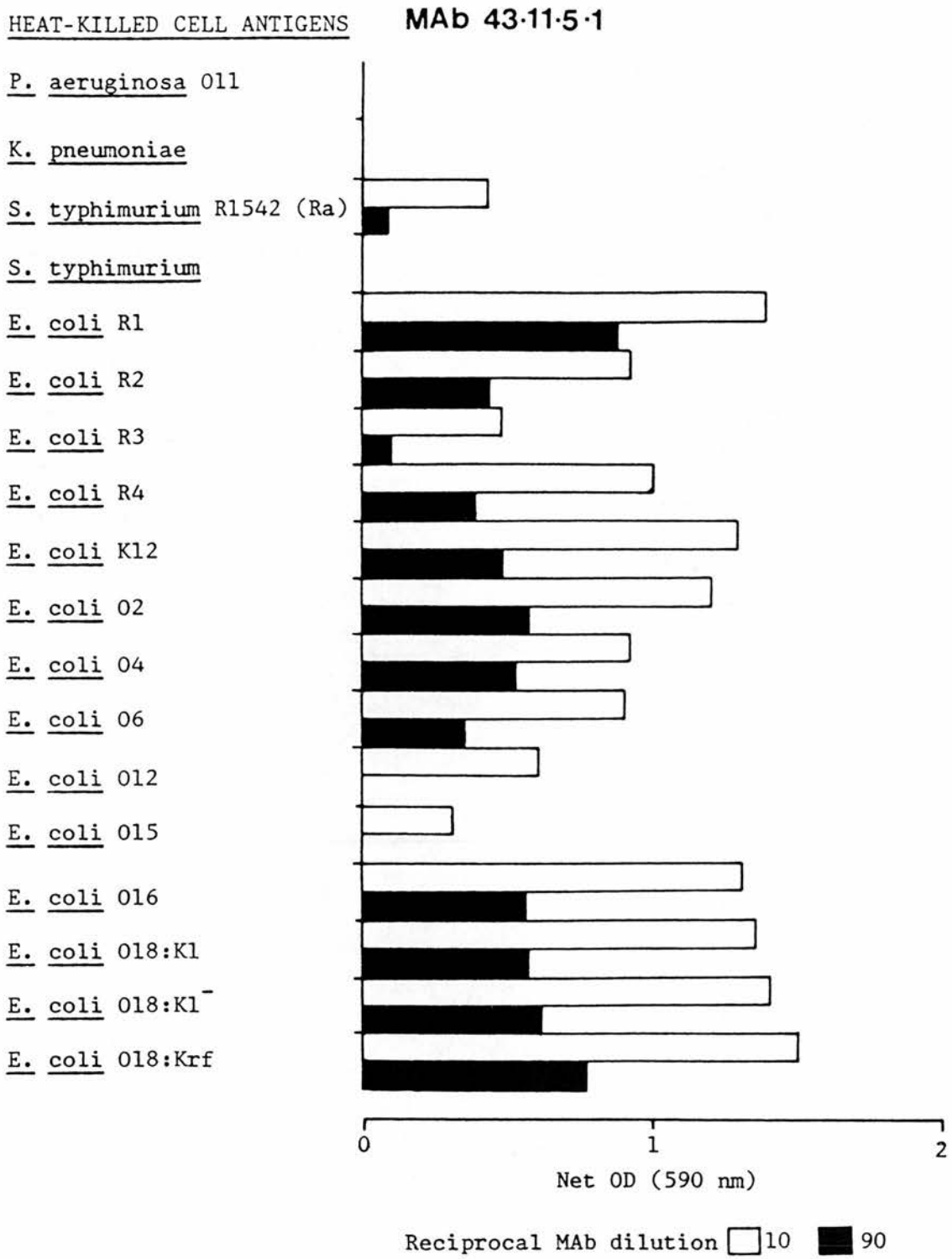


Figure 12. ELISA activity of MAb 43.11.5.1 against whole, heat-killed *E. coli* and non-*E. coli* rough and smooth cell types.

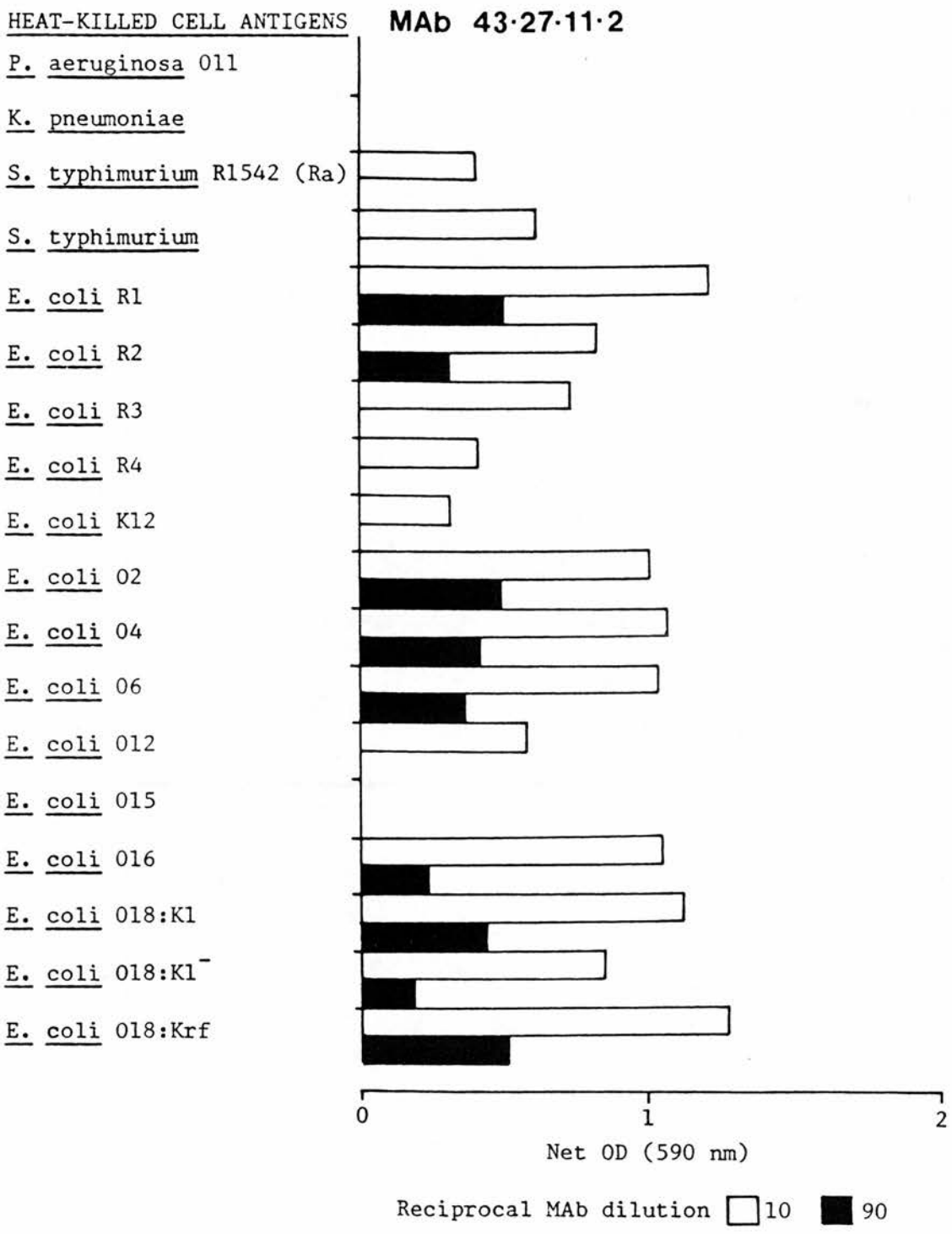


Figure 13. ELISA activity of MAb 43.27.11.2 against whole, heat-killed *E. coli* and non-*E. coli* rough and smooth cell types.

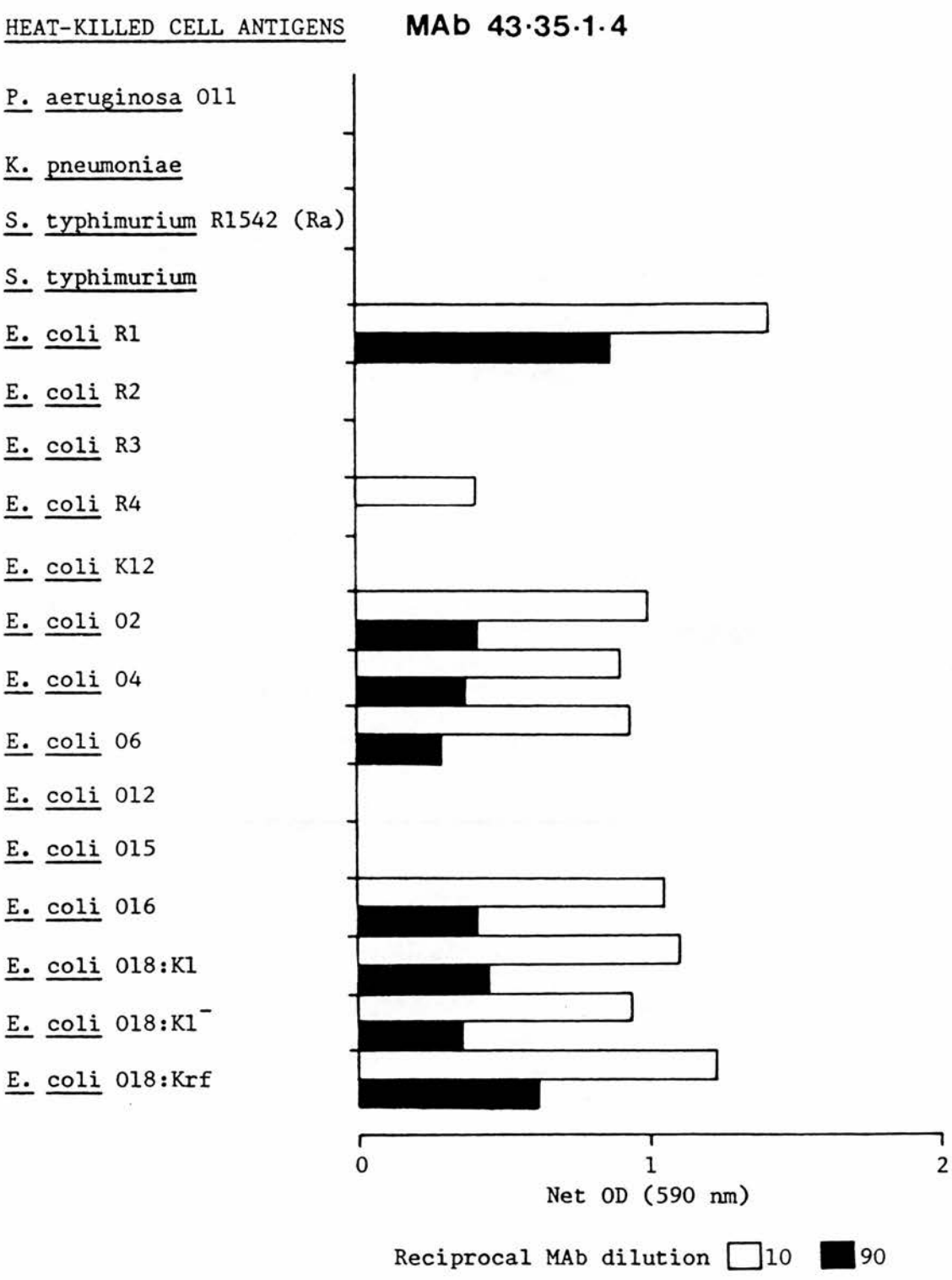


Figure 14. ELISA activity of MAb 43.35.1.4 against whole, heat-killed *E. coli* and non-*E. coli* rough and smooth cell types.

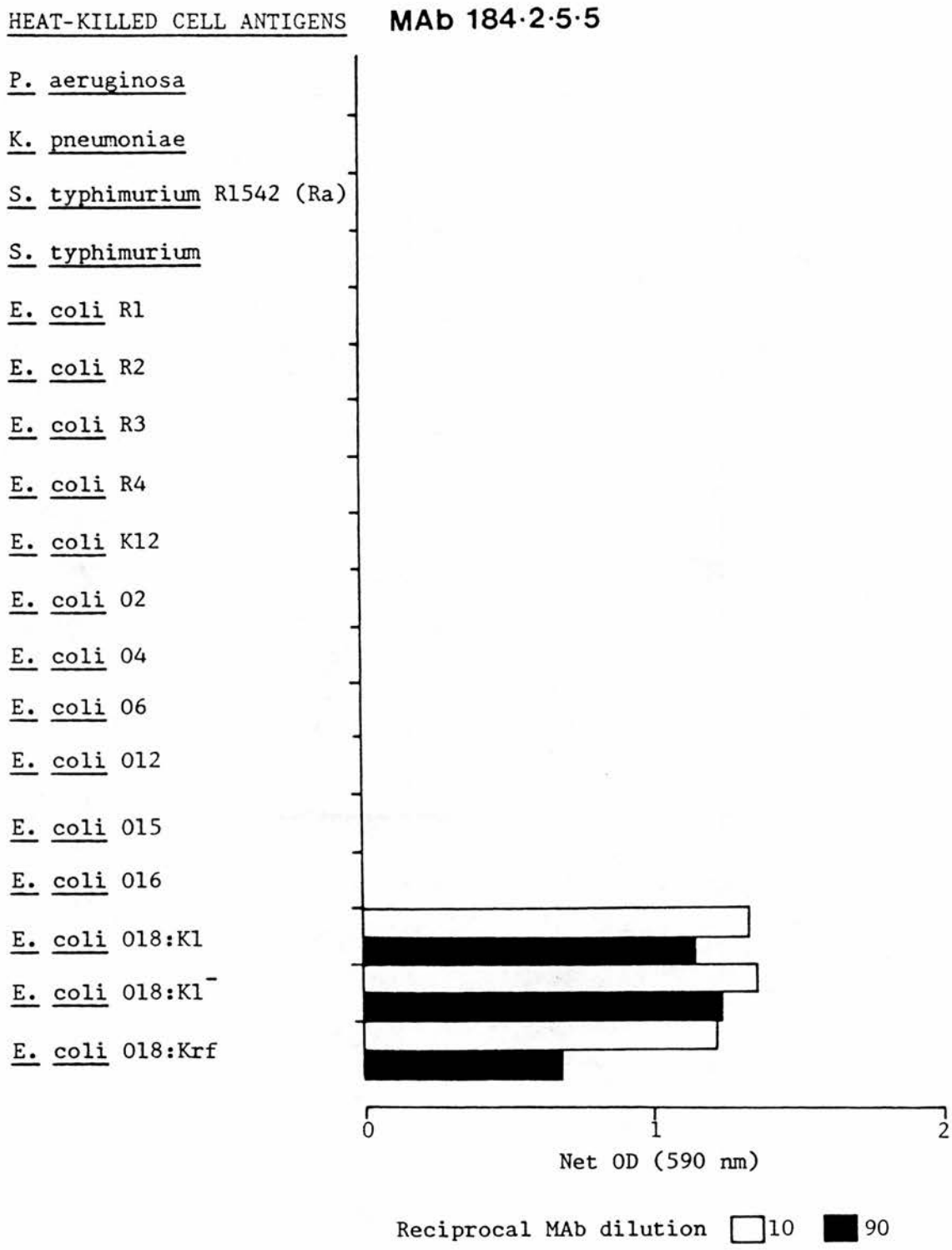


Figure 15. ELISA activity of MAb 184.2.5.5 against whole, heat-killed *E. coli* and non-*E. coli* rough and smooth cell types.

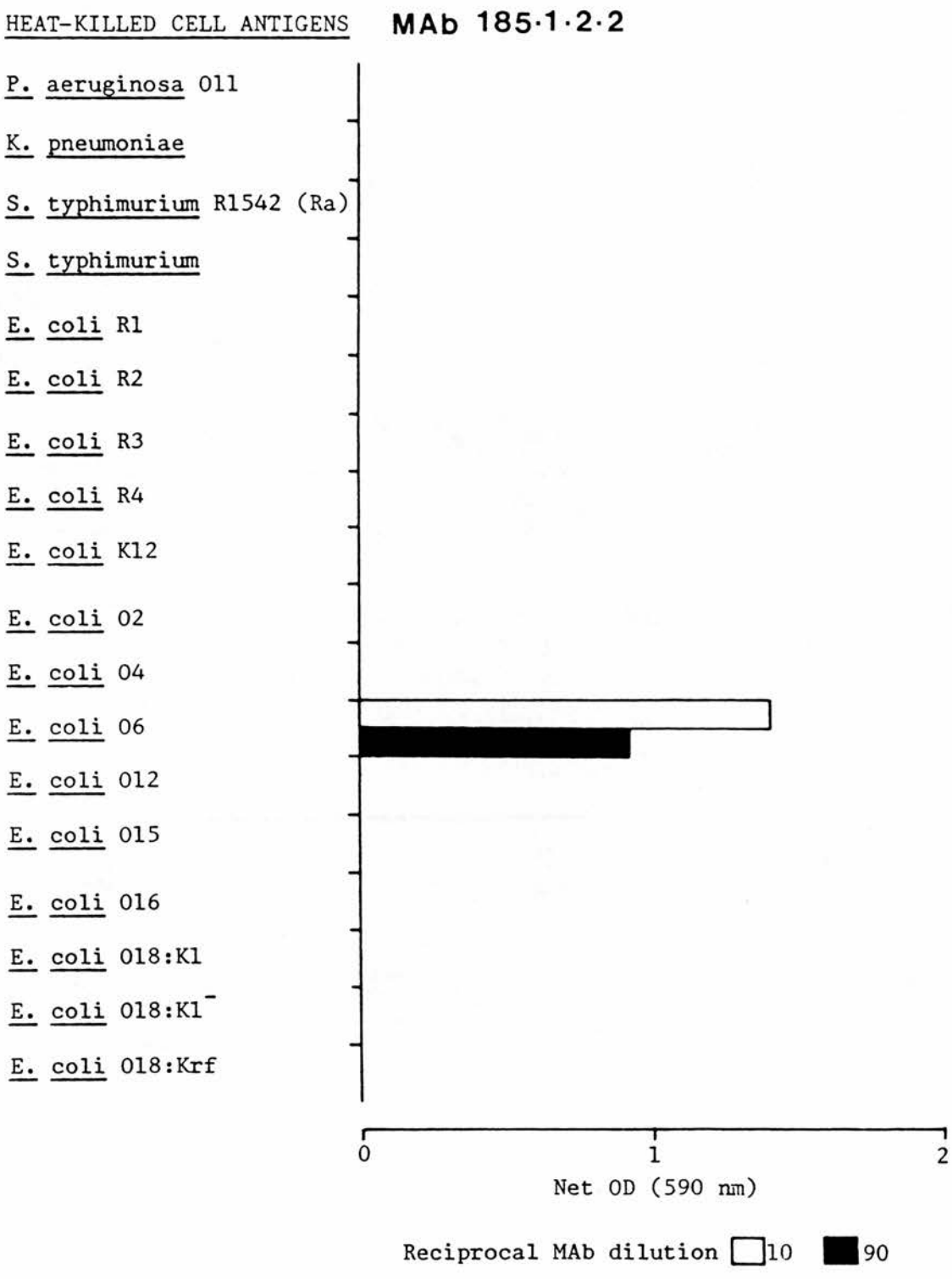


Figure 16. ELISA activity of MAb 185.1.2.2 against whole, heat-killed *E. coli* and non-*E. coli* rough and smooth cell types.

43.27.11.2 produced the weakest. MAb 43.11.5.1 reacted strongly (OD >1.0) against most *E. coli* antigens, although its binding to *E. coli* R3 and the R3 core of serotype O15 was significantly lower compared to other rough and smooth *E. coli* strains.

MAb 27.193.3 demonstrated preferential binding to the *E. coli* R3 core type and the R3 core of serotype O15 (Figure 7). Lower responses were also produced with most other heat-killed cell preparations including *P. aeruginosa* and *K. pneumoniae*. Selective binding was also observed for MAb 40.18.7.1 which preferentially bound to *Salmonella, E. coli* R2 core type and the R2 core of serotype O12 (Figure 9). Weaker reactivity was shown against *E. coli* core types R2, K12 and serotypes O2, O4. MAbs 43.3.4.8, 43.5.1.4 and 43.35.1.4 reacted almost exclusively with the *E. coli* R1 core type and those *E. coli* serotypes possessing the R1 core (Figures 10, 11 and 14). However, weak reactions against the rough cells of either, or both, R4 and K12, were shown for MAbs 43.3.4.8 and 43.35.1.4.

MAb 30.4.2.8 showed a strong affinity for the two *E. coli* O18 serotype strains (Figure 8). Weaker responses were also recorded against most other *E. coli* strains and the Gram-negative bacteria of *P. aeruginosa, K. pneumoniae* and the two *S. typhimurium* strains.

1.2 IMMUNOBLOT ANALYSIS OF ANTI-LIPOPOLYSACCHARIDE MONOCLONAL ANTIBODIES

The ELISA studies against heat-killed bacterial preparations demonstrated the wide range in cross-reactivities of the MAbs, from serotype specific to broadly cross-reactive antibodies. To confirm and investigate further the LPS binding site of each of the MAbs, immuno-

blot analysis was performed against LPS from proteinase K digests of equivalent R- and S-LPS cell types to those used in ELISA. The silver stained PAGE profiles of LPS and corresponding immunoblots of eleven MAbs are shown in Figures 17-22.

The silver stained LPS profiles of smooth strains (Figure 17a) exhibit the characteristic ladder pattern, each step up representing LPS substituted with a progressively increasing number of O-polysaccharide repeating oligosaccharide units. These were missing from the rough mutants. The immunoblot of the equivalent LPS profiles against MAb 27.150.3 (Figure 17b) demonstrated binding of the antibody to both core LPS substituted with O-antigenic side chains, and low molecular mass unsubstituted core material. The MAb was fully cross-reactive against all *E. coli* and *Salmonella* LPS preparations, but not reactive against the smooth strains of *P. aeruginosa* and *K. pneumoniae*.

Immunoblots of MAbs 27.193.3 and 30.4.2.8 are shown in Figure 18. A positive reactive band corresponding to the unsubstituted core LPS of the R-LPS mutants of *E. coli* R3 (Track 7) was observed for MAb 27.193.3 (Figure 18a). A weak response was also shown against the unsubstituted core LPS band of *E. coli* serotype O15 (possessing a R3 core type) (Track 14). The MAb was unreactive against all other LPS preparations. The high molecular mass band observed against many samples in this, and other immunoblots, is thought to represent a non-specific reaction against the proteinase K digest enzyme. In ELISA studies MAb 27.193.3 demonstrated a wider reactivity pattern against heat-killed cell preparations, although it showed preferential binding to *E. coli* R3 and O15. MAb 30.4.2.8 reacted against the ladder-like O-antigen bearing LPS bands of the two smooth *E. coli* O18 strains, and against small

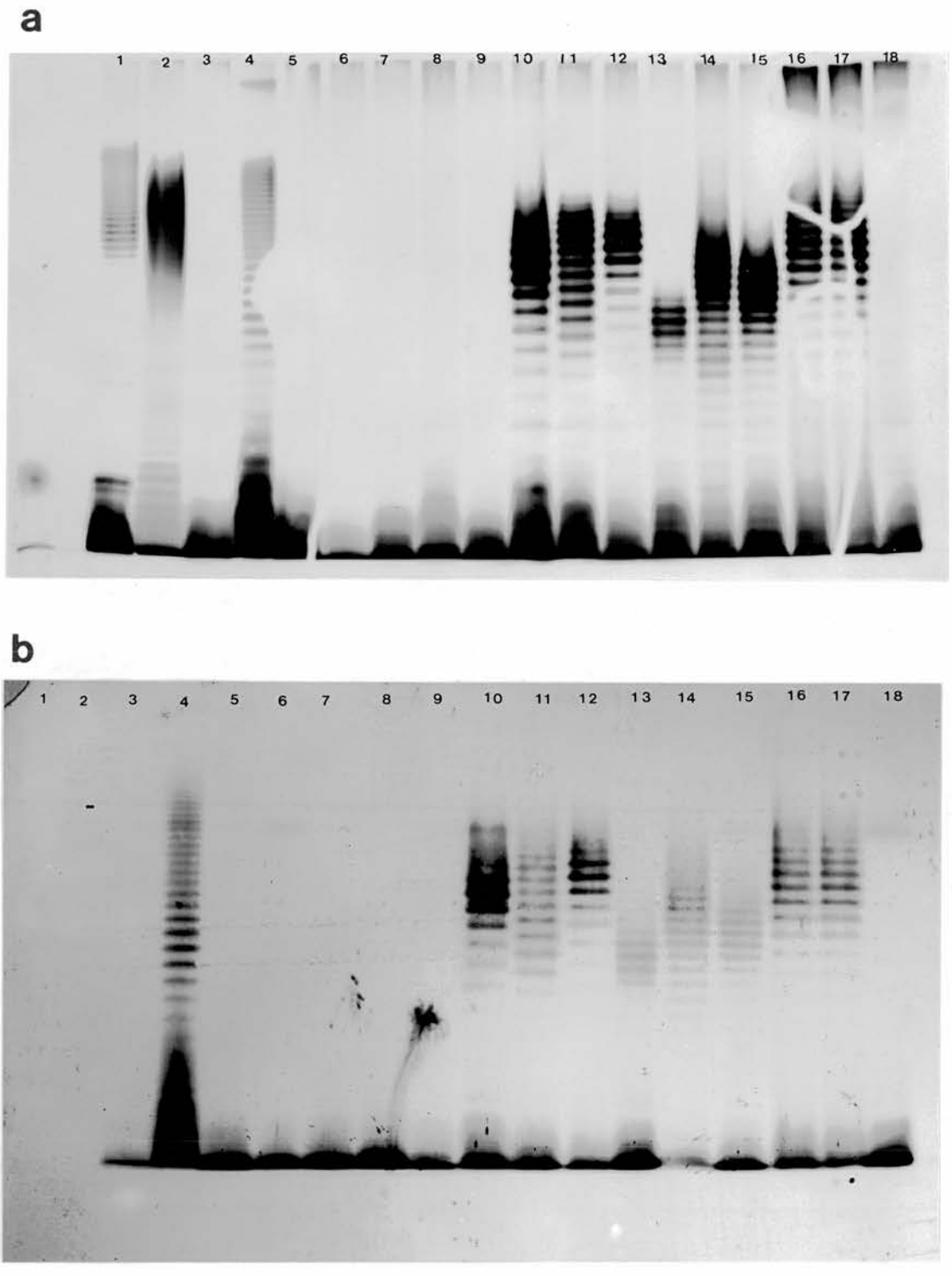


Figure 17. a) Silver stained LPS profiles of proteinase K whole cell digests of 18 *E. coli* and non-*E. coli* cell types separated by PAGE (14% w/v acrylamide). b) Immunoblot of the equivalent proteinase K whole cell digests separated by PAGE (14% w/v acrylamide) followed by transfer to NIC paper and probed with MAb 27.150.3 (supernatant 1:10). Track 1, *P. aeruginosa* O11 (S-LPS); Track 2, *K. pneumoniae* (S-LPS); Track 3, *S. typhimurium* R1542, Ra (R-LPS); Track 4, *S. typhimurium* (S-LPS); Tracks 5-9, *E. coli* R1, R2, R3, R4 and K12 (R-LPSs); Tracks 10-17, *E. coli* serotype strains O2, O4, O6, O12, O15, O16, O18:K1 and O18:K1- (S-LPSs) and Track 18, *E. coli* O18:Krf (R-LPS).

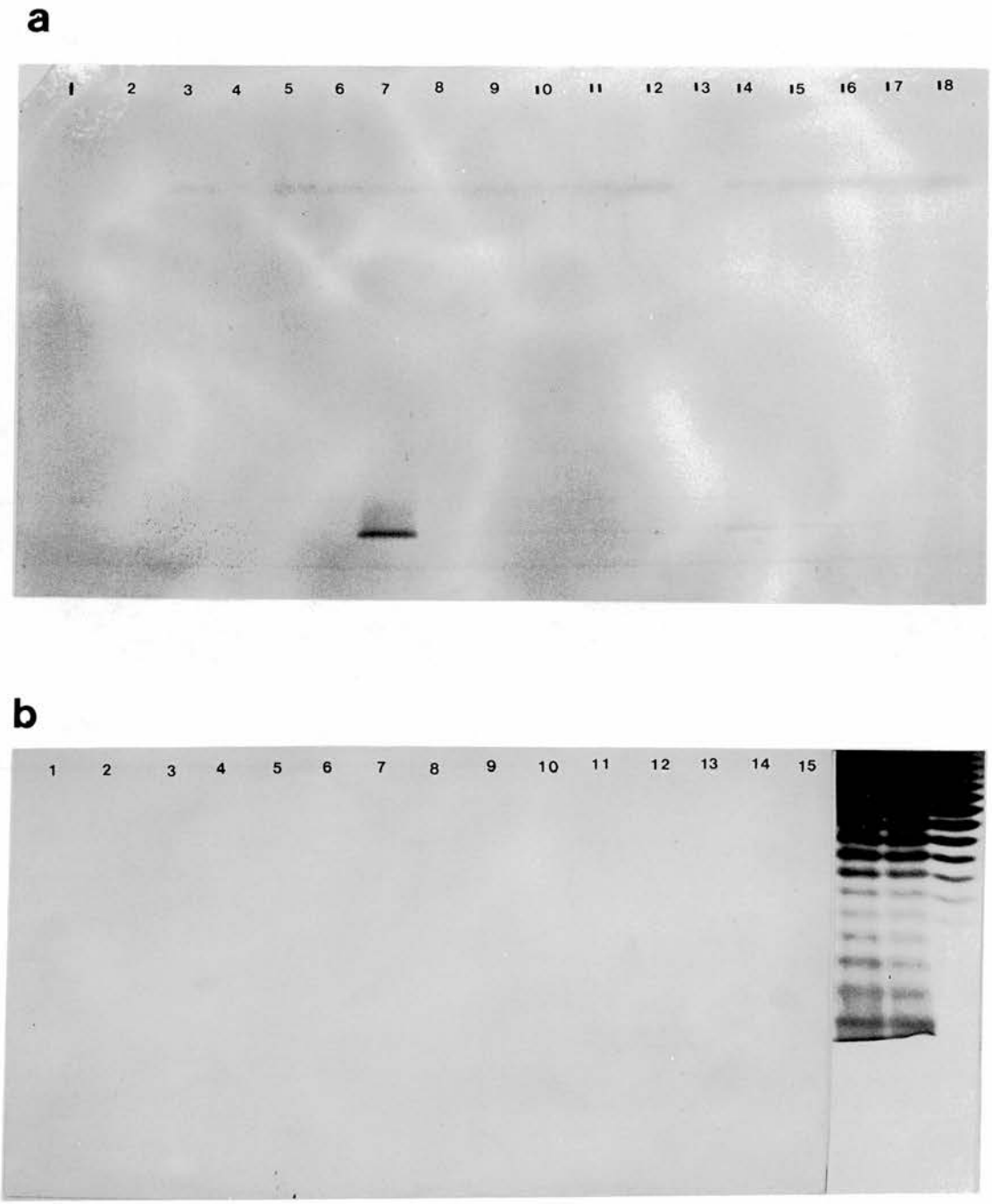


Figure 18. Immunoblots of proteinase K whole cell digests of 18 *E. coli* and non-*E. coli* cell types separated by PAGE (14% w/v acrylamide) followed by transfer to NIC paper and probed with MAbs a) 27.193.3 and b) 30.4.2.8 (supernatant 1:10). Track 1, *P. aeruginosa* O11 (S-LPS); Track 2, *K. pneumoniae* (S-LPS); Track 3, *S. typhimurium* R1542, Ra (R-LPS); Track 4, *S. typhimurium* (S-LPS); Tracks 5-9, *E. coli* R1, R2, R3, R4 and K12 (R-LPSs); Tracks 10-17, *E. coli* serotype strains O2, O4, O6, O12, O15, O16, O18:K1 and O18:K1- (S-LPSs) and Track 18, *E. coli* O18:Krf (R-LPS).

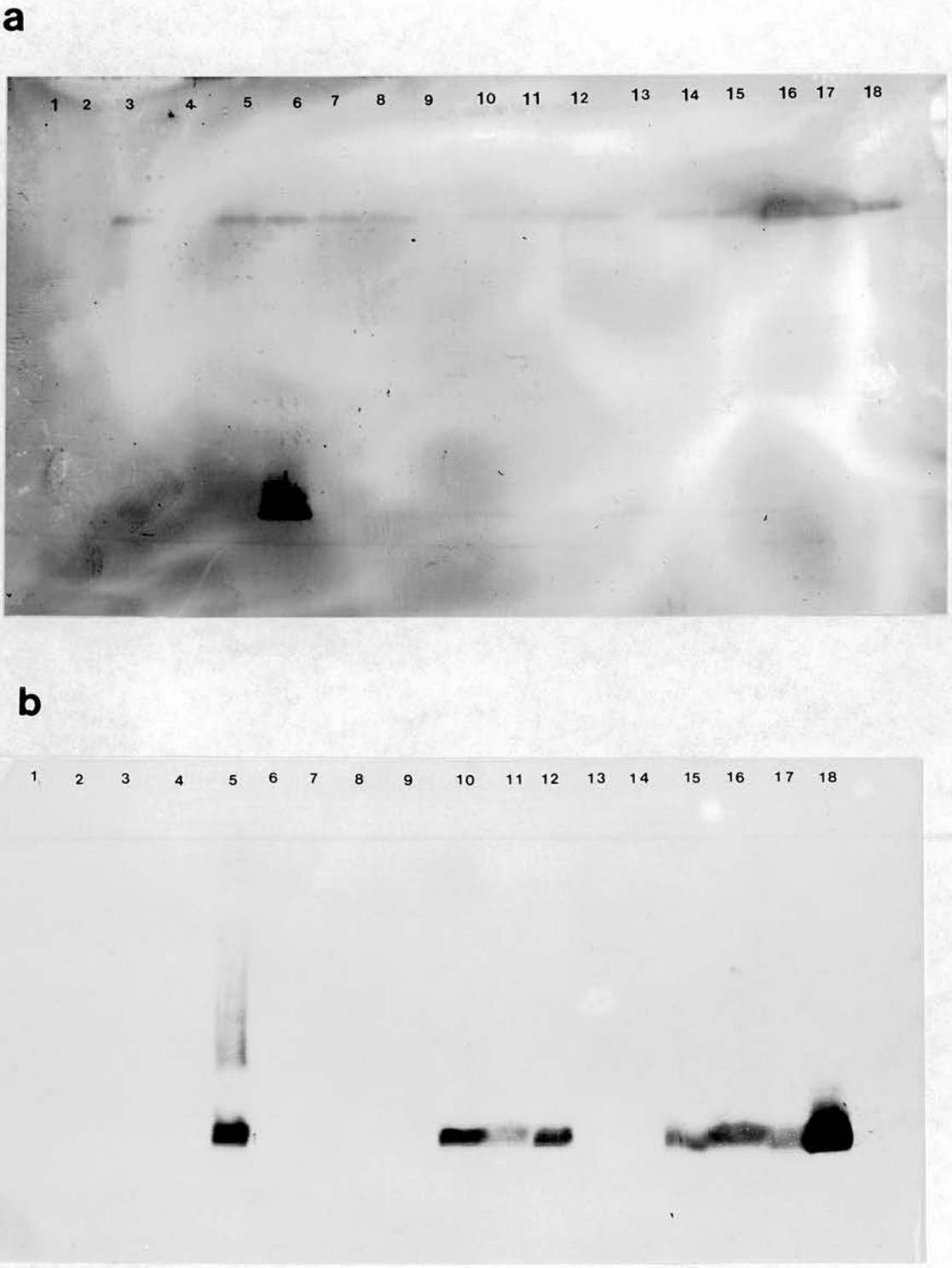


Figure 19. Immunoblots of proteinase K whole cell digests of 18 *E. coli* and non-*E. coli* cell types separated by PAGE (14% w/v acrylamide) followed by transfer to NIC paper and probed with MAbs a) 40.18.7.1 and b) 43.3.4.8 (supernatant 1:10). Track 1, *P. aeruginosa* 011 (S-LPS); Track 2, *K. pneumoniae* (S-LPS); Track 3, *S. typhimurium* R1542, Ra (R-LPS); Track 4, *S. typhimurium* (S-LPS); Tracks 5-9, *E. coli* R1, R2, R3, R4 and K12 (R-LPSs); Tracks 10-17, *E. coli* serotype strains 02, 04, 06, 012, 015, 016, 018:K1 and 018:K1- (S-LPSs) and Track 18, *E. coli* 018:Krf (R-LPS).

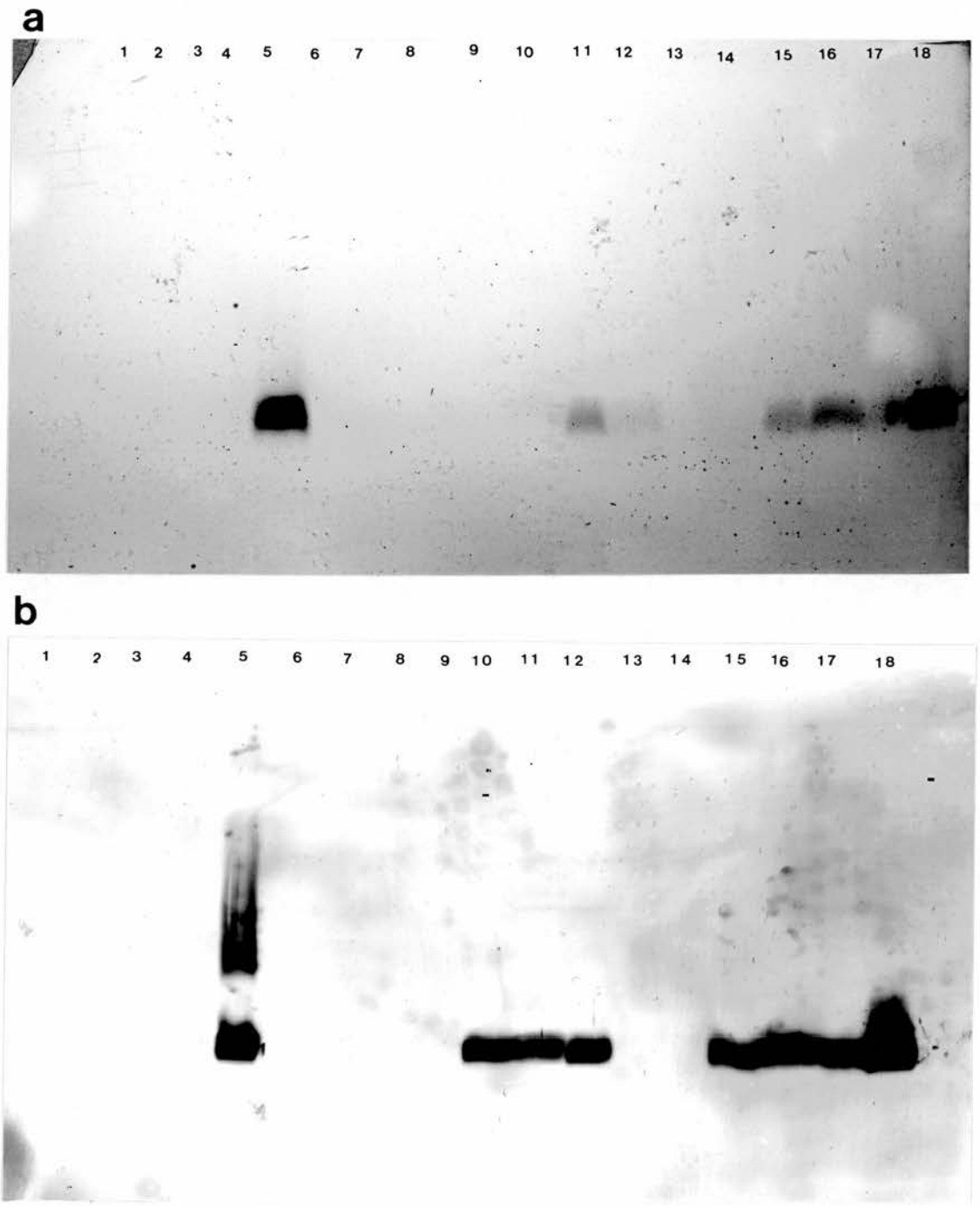


Figure 20. Immunoblots of proteinase K whole cell digests of 18 *E. coli* and non-*E. coli* cell types separated by PAGE (14% w/v acrylamide) followed by transfer to NIC paper and probed with MAbs a) 43.5.1.4 and b) 43.11.5.1 (supernatant 1:10) Track 1, *P. aeruginosa* O11 (S-LPS); Track 2, *K. pneumonia* (S-LPS); Track 3, *S. typhimurium* R1542, Ra (R-LPS); Track 4, *S. typhimurium* (S-LPS); Tracks 5-9, *E. coli* R1, R2, R3, R4 and K12 (R-LPSs); Tracks 10-17, *E. coli* serotype strains O2, O4, O6, O12, O15, O16, O18:K1 and O18:K1- (S-LPSs) and Tack 18, *E. coli* O18:Krf (R-LPS).

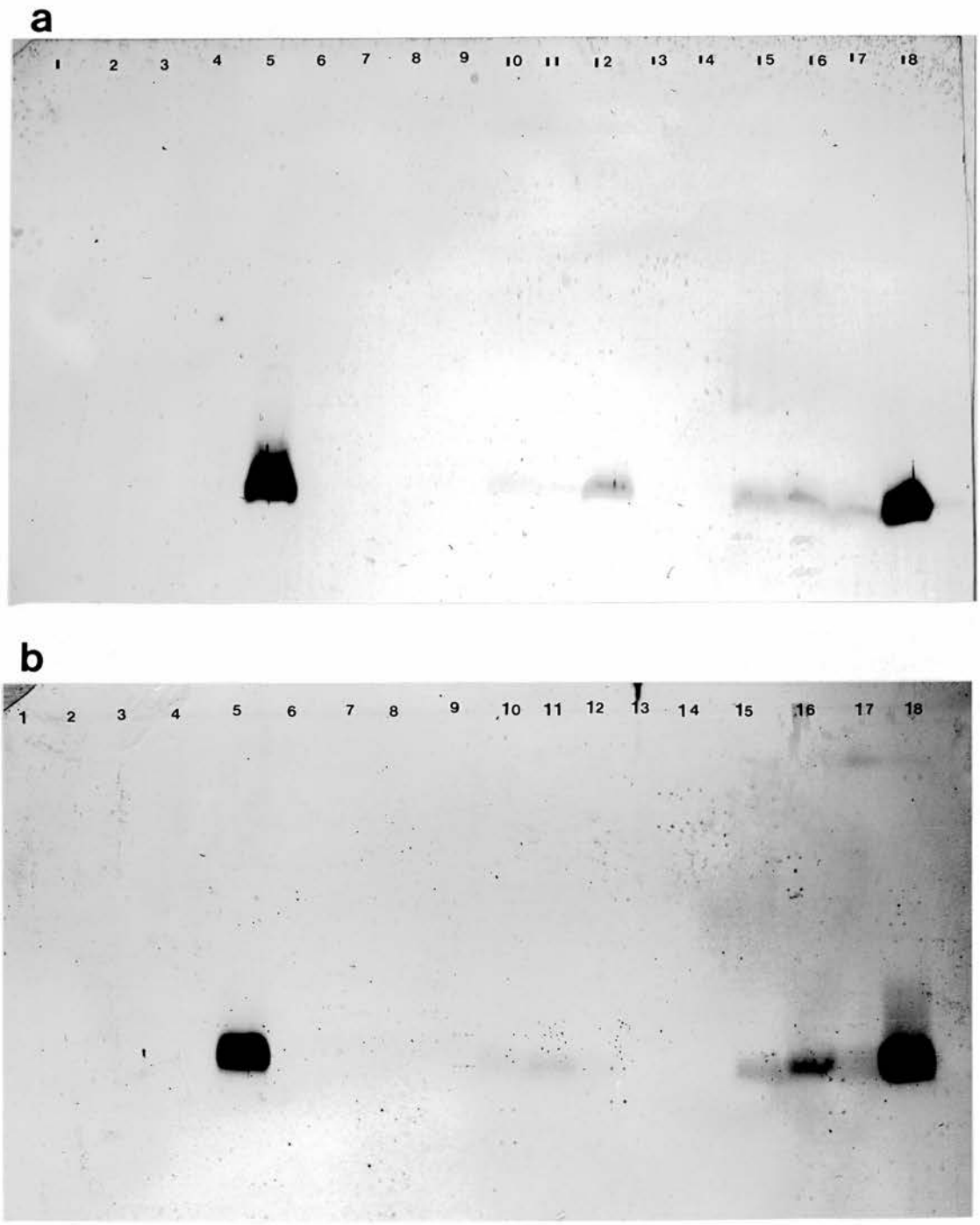


Figure 21. Immunoblots of proteinase K whole cell digests of 18 *E. coli* and non-*E. coli* cell types separated by PAGE (14% w/v acrylamide) followed by transfer to NIC paper and probed with MAbs a) 43.27.11.2 and b) 43.35.1.4 (supernatant 1:10). Track 1, *P. aeruginosa* O11 (S-LPS); Track 2, *K. pneumoniae* (S-LPS); Track 3, *S. typhimurium* R1542, Ra (R-LPS); Track 4, *S. typhimurium* (S-LPS); Tracks 5-9, *E. coli* R1, R2, R3, R4 and K12 (R-LPSs); Tracks 10-17, *E. coli* serotype strains O2, O4, O6, O12, O15, O16, O18:K1 and O18:K1- (S-LPSs) and Track 18, *E. coli* O18:Krf (R-LPS).

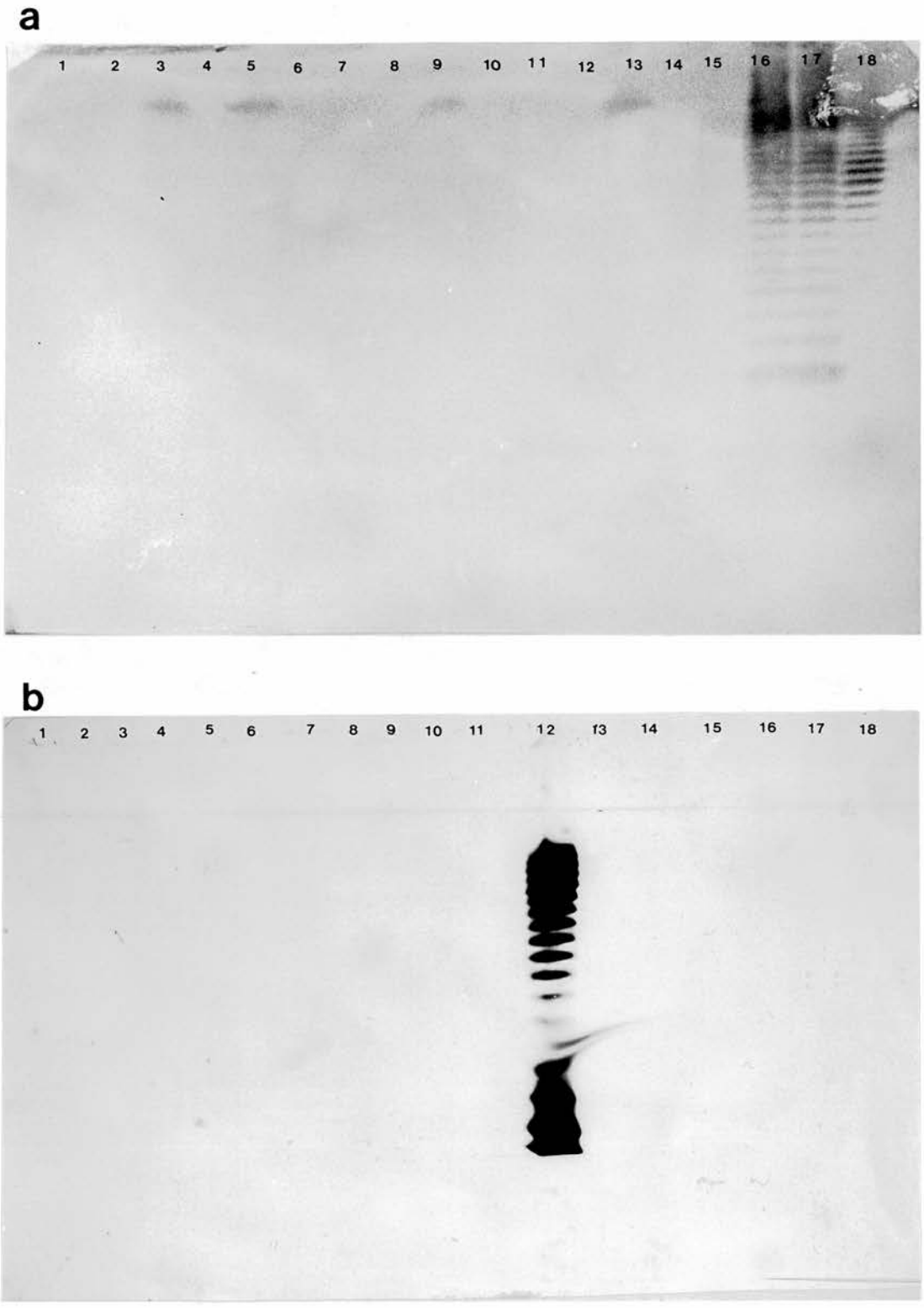


Figure 22. Immunoblots of proteinase K whole cell digests of 18 *E. coli* and non-*E. coli* cell types separated by PAGE (14% w/v acrylamide) followed by transfer to NIC paper and probed with MAbs a) 184.2.5.5 and b) 185.1.2.2 (supernatant 1:10). Track 1, *P. aerugonosa* O11 (S-LPS); Track 2, *K. pneumoniae* (S-LPS); Track 3, *S. typhimurium* R1542, Ra (R-LPS); Track 4, *S. typhimurium* (S-LPS); Tracks 5-9, *E. coli* R1, R2, R3, R4 and K12 (R-LPSs); Tracks 10-17, *E. coli* serotype strains O2, O4, O6, O12, O15, O16, O18:K1 and O18:K1- (S-LPSs) and Track 18, *E. coli* O18:Krf (R-LPS).

amounts of high molecular mass O-antigen leaking from the rough mutant of O18 (Tracks 16-18) (Figure 18b). Whilst showing preferential binding to the two *E. coli* O18 serotypes, ELISA studies again demonstrated a broader reactivity against other LPSs.

MAb 40.18.7.1 reacted against the unsubstituted core LPS of the R-LPS mutant of R2 (Track 6) and very weakly against the unsubstituted core LPS region of *E. coli* serotype O12 (possessing a R2 core type) (Track 13) (Figure 19a). The MAb was also reactive against the full core R-LPS mutant (Ra) of *S. typhimurium* R1542, although the band is barely visible above the background on the photograph shown.

The immunoblots of MAbs 43.3.4.8, 43.5.1.4, 43.11.5.1, 43.27.11.2 and 43.5.1.4 are shown in Figures 19, 20 and 21. All MAbs reacted against the low molecular mass core-glycolipid band, corresponding to the unsubstituted LPS of both rough and smooth *E. coli* strains possessing the R1 core structure. The strongest reactions in all these immunoblots were against the core R-LPS mutants of *E. coli* R1 and O18:Krf (Tracks 5 and 18), whilst MAbs 43.3.4.8 (Figure 19b) and 43.11.5.1 (Figure 20b) produced the strongest overall reactions against both R- and S-LPSs. ELISA activities of these MAbs (especially 43.11.5.1 and 43.27.11.2) again demonstrated greater cross-reactivity than that shown in immunoblots.

The specificities of MAbs 184.2.5.5 and 185.1.2.2 for the O-serotypes of *E. coli* O18 and *E. coli* O6 respectively, was confirmed by immunoblotting. MAb 184.2.5.5 reacted strongly against the O-antigen bearing molecular mass bands of both *E. coli* O18 strains and the high molecular mass O-antigen leaking from the rough mutant of O18 (Tracks 16-18)

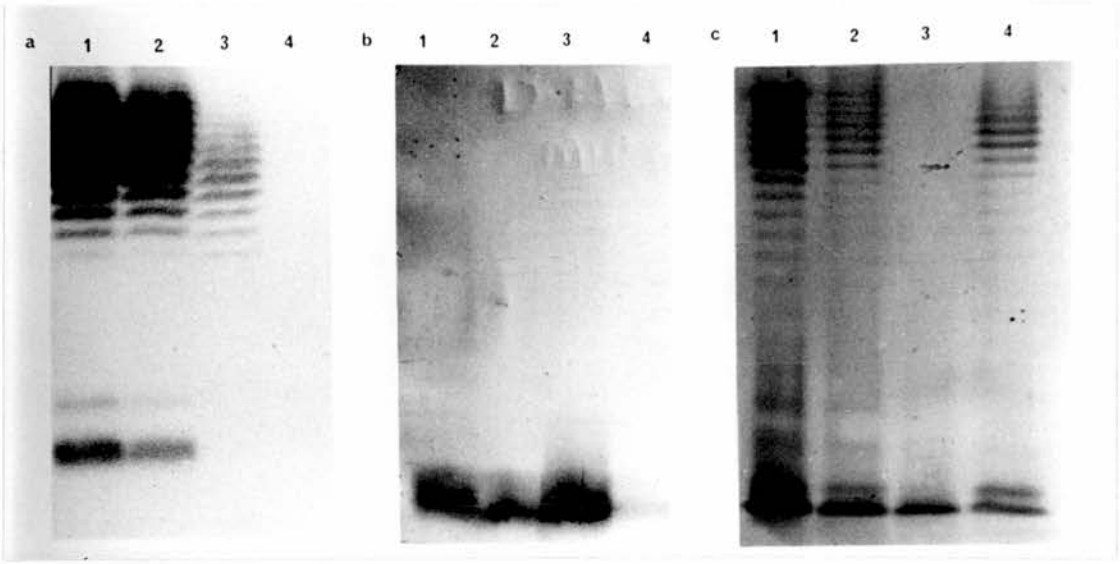


Figure 23. Immunoblots of proteinase K whole cell digests of 4 *E. coli* strains probed with MAbs 184.2.5.5 (a); 43.27.11.2 (b) and 27.150.3 (c) (supernatant 1:10). Track 1, O18:K1 (S-LPS); Track 2, O18:K1 (S-LPS); Track 3, O18:Krf (R-LPS) and Track 4, O6:K5 (S-LPS).

(Figure 22a). MAb 185.1.2.2 reacted strongly against the corresponding O-antigen bearing molecular mass bands of *E. coli* O6 (Figure 22b).

Immunoblots probed with three MAbs described above are shown in Figure 23 as a direct comparison of their binding specificities. Serotype specific MAb 184.2.5.5 reacted with only substituted core material bearing O-antigen LPS (Figure 23a). Of the two anti-core MAbs, 43.27.11.2 bound only to the fast migrating species that corresponded to the unsubstituted core-glycolipid, whilst 27.150.3 reacted with both the low molecular mass band of unsubstituted core LPS and substituted core material of higher molecular mass bands (Figure 23b and c).

1.3 FLOW CYTOMETRIC ANALYSIS OF ANTI-LIPOPOLYSACCHARIDE MONOCLONAL ANTIBODIES AGAINST WHOLE BACTERIA

Table 5 shows the binding activities of anti-LPS MAbs against whole cells in suspension by flow cytometry. Results are presented as percentage values, which refer to the proportion of cells exhibiting positive fluorescence above a background staining level set at 1% ± 0.5%. The serotype specific MAbs of 184.2.5.5 and 185.1.2.2 showed strong positive fluorescence (>50%) against the O-antigens of *E. coli* O18 and O6 respectively. No reactivity was observed with whole cells of all other Gram-negative cells.

The specificities of those MAbs reactive against core-glycolipid LPS were confirmed by results of flow cytometry. Those MAbs previously shown to preferentially bind to selective *E. coli* core R-LPSs, also demonstrated strong affinity against their respective homologous whole cells (for example, MAbs 27.193.3, 40.18.7.1 and 43.3.4.8). However, with the exception of O-serotype reactive MAbs, MAbs either demon-

Table 5. Flow cytometric analysis of 11 anti-LPS MAbs binding to rough and smooth whole cells of *E. coli*, and *S. typhimurium* R1542. Percentage values represent the mean positive fluorescence of bacteria above background levels.

	Percentage of cells showing positive fluorescence								
	E. coli core type					*E. coli* O-serotype			*S. typhimurium*
MAb	R1	R2	R3	R4	K12	O6	O12	O15	O18	R1542
27.150.3	31	27	15	18	27	ND	3	0	2	10
27.193.3	0	18	48	14	19	ND	0	12	0	13
30.4.2.8	21	18	8	30	22	ND	0	0	56	11
40.18.7.1	21	52	5	13	29	ND	12	0	0	45
43.3.4.8	76	0	0	16	0	ND	0	0	34	0
43.5.1.4	69	0	0	0	0	ND	0	0	28	0
43.11.5.1	66	63	3	34	31	ND	9	0	15	7
43.27.11.2	65	35	3	24	29	ND	0	0	3	9
43.35.1.4	72	0	0	12	0	ND	0	0	20	0
184.2.5.5	0	0	0	0	0	0	0	0	75	0
185.1.2.2	0	0	0	0	0	69	0	0	0	0

ND = not done

Table 6. Flow cytometric analysis of anti-LPS MAb binding to *E. coli* O18:K1 : effect of boiling bacteria.

MAb	Percentage of cells showing positive fluorescence *	
	Unboiled	Boiled **
27.150.3	3 (±2)	39 (±4)
43.3.4.8	28 (±6)	57 (±11)
43.11.5.1	18 (±5)	71 (±9)
43.27.11.2	5 (±2)	46 (±8)
184.2.5.5	74 (±8)	71 (±12)
185.1.2.2	0	0

* Each FC value represents the mean (±SD) from three separate experiments.

** Bacteria were boiled for 15 min before incubation with MAb and staining with FITC-labelled secondary antibody.

strated no reactivity or weak reactivity against smooth *E. coli* strains (most showing labelling of ≤20%). Those MAbs reacting against selective smooth whole cells at levels of >20% included MAbs 43.3.4.8 and 43.5.1.4. MAb 27.150.3, shown previously to be cross-reactive in ELISA and immunoblotting studies was again reactive against all *E. coli* core types, although weak labelling (<10%) was associated with smooth *E. coli* strains.

The failure of anti-core-glycolipid MAbs to react strongly against intact bacteria suggests an inability of these MAbs to recognize clearly their respective epitopes on smooth, wild type bacteria. The effect of boiling bacteria for 15 min on recognition by selected MAbs was investigated by subjecting boiled and unboiled *E. coli* O18:K1 to flow cytometric analysis. MAbs reactive against core LPS demonstrated significant increases in binding to boiled compared to unboiled bacteria (Table 6). Samples probed with the negative control MAb 185.1.2.2 (specific for *E. coli* O6 serotype) produced no detectable positive fluorescence. *E. coli* O18 serotype specific MAb, 184.2.5.5, exhibited equally high levels of binding to boiled and unboiled bacteria.

1.4 SEQUENTIAL ABSORPTIONS OF MONOCLONAL ANTIBODIES BY WHOLE BACTERIA OF SALMONELLA LIPOPOLYSACCHARIDE CHEMOTYPES

The binding specificities of those MAbs reactive against the core-glycolipid component of the LPS molecule were further studied by sequential absorption with whole bacteria of *S. typhimurium* chemotypes (Ra, Rc, Re) and lipid A (Figures 24 and 25). The residual ELISA reactivity of each MAb after each absorption stage was measured against selected purified LPS. The percentage residual ELISA reactivity of the

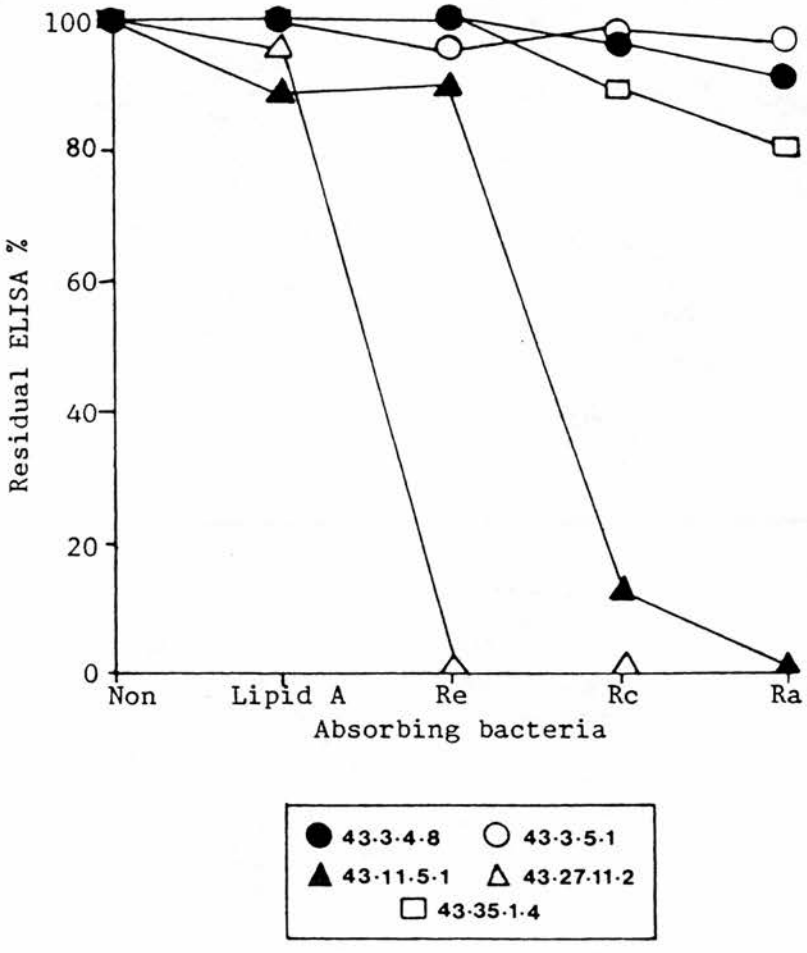


Figure 24. Serial absorption of 5 MAbs with different chemotypes of rough *S. typhimurium* bacteria, used sequentially in an ascending order of LPS size. Residual ELISA reactivity to *E. coli* R1 (R-LPS) at each stage of absorption are expressed as a percentage of the reactivity of unabsorbed MAb.

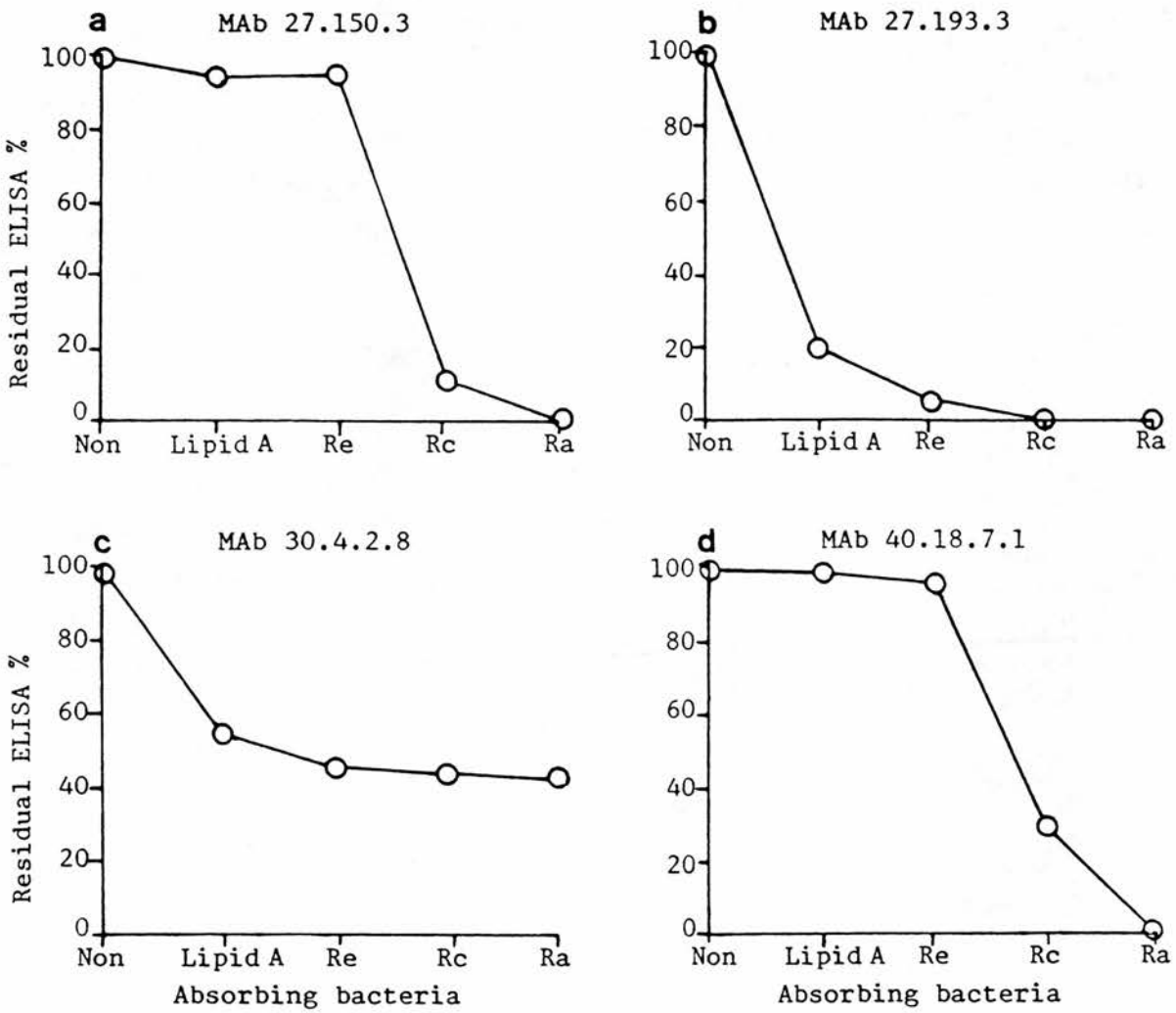


Figure 25. Serial absorption of 4 MAbs with different chemotypes of rough *S. typhimurium* whole bacteria, used sequentially in an ascending order of LPS size. Residual ELISA reactivity to *E. coli* R1 (R-LPS) (for MAb 27.150.3); *E. coli* R3 (R-LPS) (for MAb 27.193.3); *E. coli* O18 (S-LPS) (for MAb 30.4.2.8), and *E. coli* R2 (R-LPS) (for MAb 40.18.7.1) at each stage of absorption are expressed as a percentage of the reactivity of unabsorbed MAb.

five MAbs from fusion 43 after each absorption are shown in Figure 24. MAb 43.27.11.2 was absorbed out on Re bacteria while MAb 43.11.5.1 was primarily absorbed out on Rc bacteria. All other MAbs from this set showed only minor reductions in ELISA reactivity, even at the end of the absorption sequence with the complete Ra core.

The percentage residual ELISA reactivity of four other MAbs after each absorption are shown in Figure 25. Significant reductions in residual ELISA reactivity occurred for MAbs 27.150.3 and 40.18.7.1 following the Rc absorption stage. MAb 27.193.3 was readily absorbed on the lipid A acid hydrolysed bacteria of *S. typhimurium* 1102 Re. Serial absorption of MAb 30.4.2.8 showed incomplete absorption by lipid A to 56% residual ELISA reactivity. After absorption with Re bacteria, reactivity stabilized at 44% for the remainder of the absorption sequence.

1.5 DEVELOPMENT OF A PROTEINASE K EXTRACTED LIPOPOLYSACCHARIDE ELISA

The reactivities of individual MAbs were shown to differ between immunoblotting and ELISA studies. Although some MAbs demonstrated restrictive specificity against proteinase K extracted LPS in immunoblots, they appeared more cross-reactive against heat-killed cells in ELISA. An ELISA employing proteinase K extracted LPS was developed to compare the binding activities of MAbs in this assay with reactivities against similarly prepared antigens in immunoblotting. Details of the digestion of cells and coating to microplates are described in Materials and Methods.

In preliminary experiments the amount of proteinase K enzyme required to digest a heat-killed suspension of cells, set to an A_{525} of 1.0, was investigated. A minimum volume of 10 μl proteinase K solution (2.5 mg

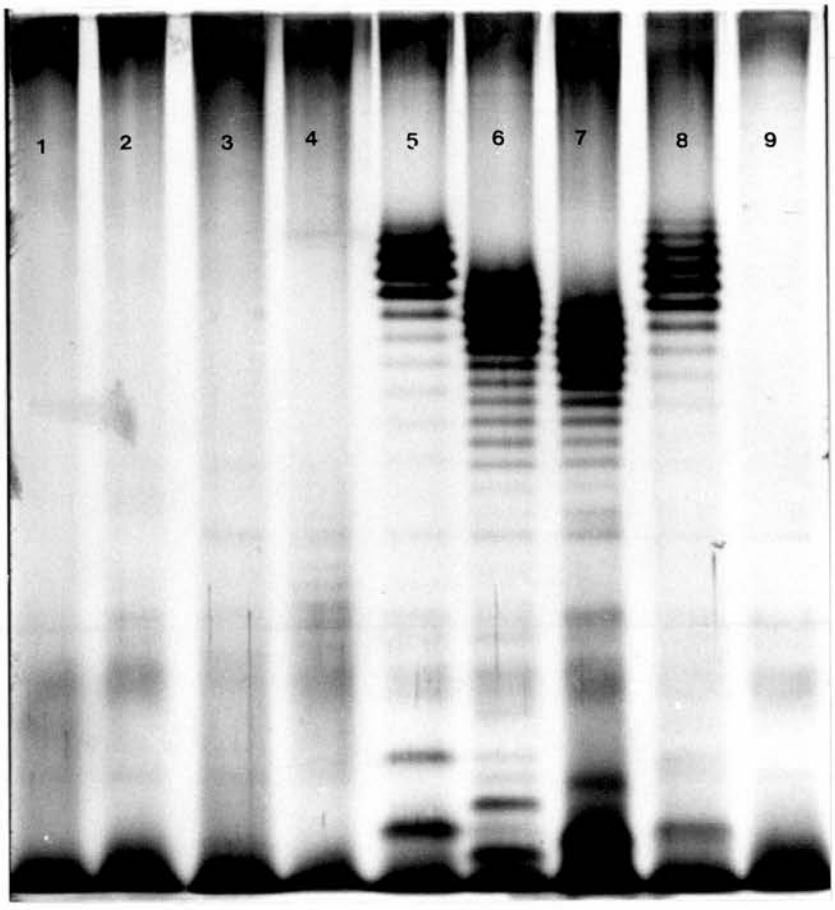


Figure 26. Silver stained LPS profiles of proteinase K whole cell digests (prepared by a modification of the conventional proteinase K digestion method for coating ELISA microplates) of 9 *E. coli* R- and S-LPS strains separated by PAGE (14% w/v acrylamide). Tracks 1-4, *E. coli* R1, R2, R3, R4 (R-LPSs), Tracks 5-8, *E. coli* serotypes O6, O15, O16 and O18 (S-LPSs) and Track 9, *E. coli* O18:Krf (R-LPS).

ml^{-1}) per 1.5 ml cell suspension was found to digest the cells effectively. Digested samples from several R- and S-LPS *E. coli* strains were analysed on silver stained gels, loaded with 40 μl of an equal volume of digested sample and double-strength sample buffer (Figure 26). Samples were shown to contain LPS as the only major macromolecular component revealed by silver staining. Smooth strains demonstrated their characteristic ladder pattern, whilst rough strains contained a low molecular mass band corresponding to unsubstituted core LPS. A qualitative comparison of the staining intensity of known concentrations of purified LPS with dilutions of proteinase K extracted LPS indicated that 20 μl of proteinase K LPS was equivalent to approximately 5 μg of purified LPS.

Scott & Barclay (1987) complexed LPS with the cationic polypeptide polymyxin B to attain stable binding of purified R-LPS to microplates, for the detection of natural antibodies in human sera to R-LPS. In an attempt to develop a stable, reproducible ELISA employing proteinase K extracted LPS, samples were complexed with polymyxin by a similar method as described in Materials and Methods (Page 99).

Optimization of coating of LPS-polymyxin complexes for ELISA

Microplates were coated with doubling dilutions of polymyxin-O18 (S-LPS) or R1 (R-LPS) complexes from 1:5 to 1:80. Two anti-LPS MAbs, an O18-antigen specific MAb 184.2.5.5 and an anti-core specific MAb 27.150.3 were titrated against the R- and S-LPS respectively (Figure 27). The maximum response at each MAb dilution was generally obtained at a coating complex dilution of 1:10 for both S- and R-LPS. Positive reactions were detectable for both 1:10 and 1:20 complex dilutions at a MAb dilution of 1:2560.

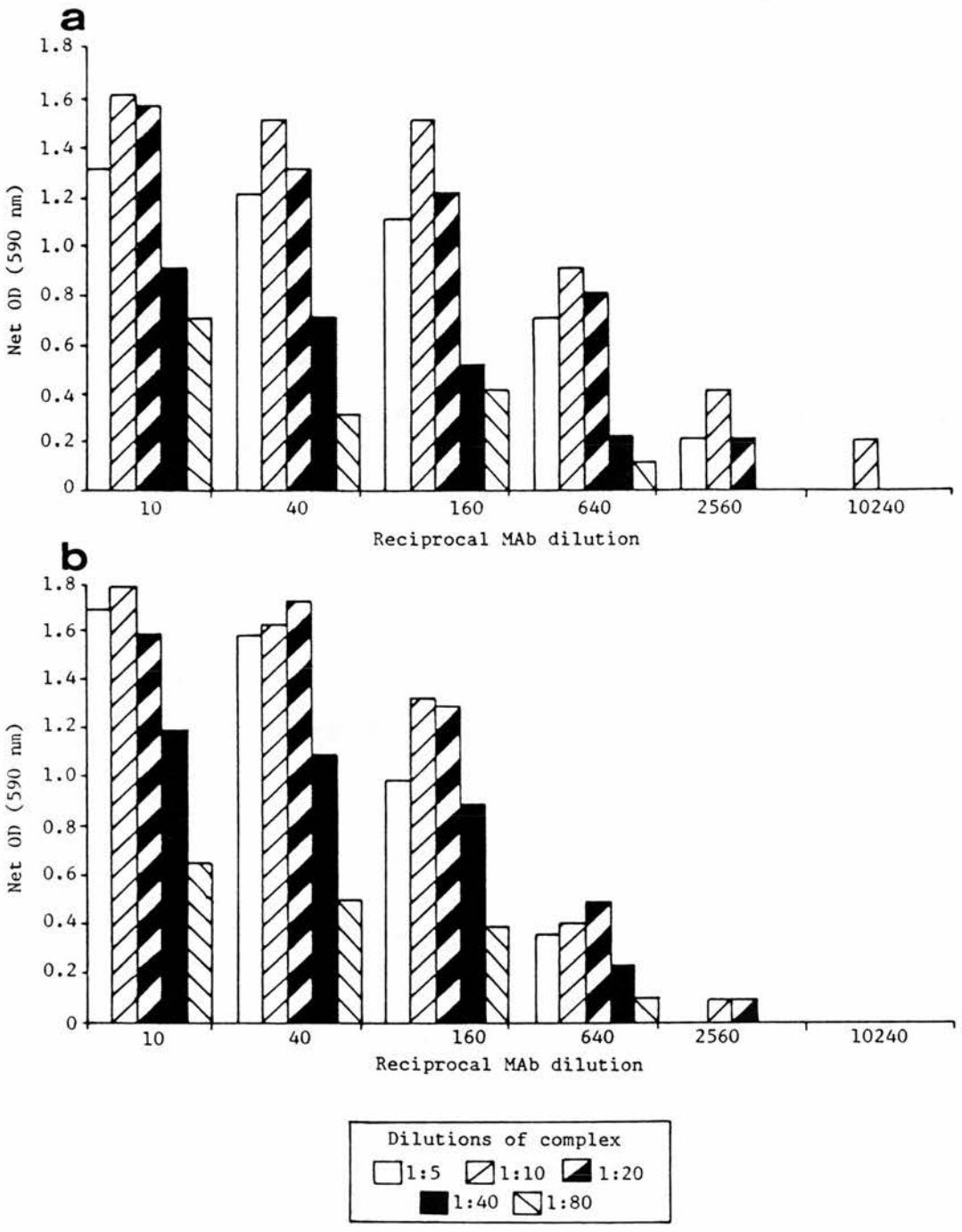


Figure 27. ELISA activity of 2 anti-LPS MAbs against doubling dilutions of coated proteinase K extracted LPS polymyxin complexes.

a) Anti-core specific MAb 27.150.3 against *E. coli* R1 extracted LPS

b) Anti-O18 specific MAb 184.2.5.5 against *E. coli* O18 extracted LPS

Each value represents the mean optical density value of triplicate readings.

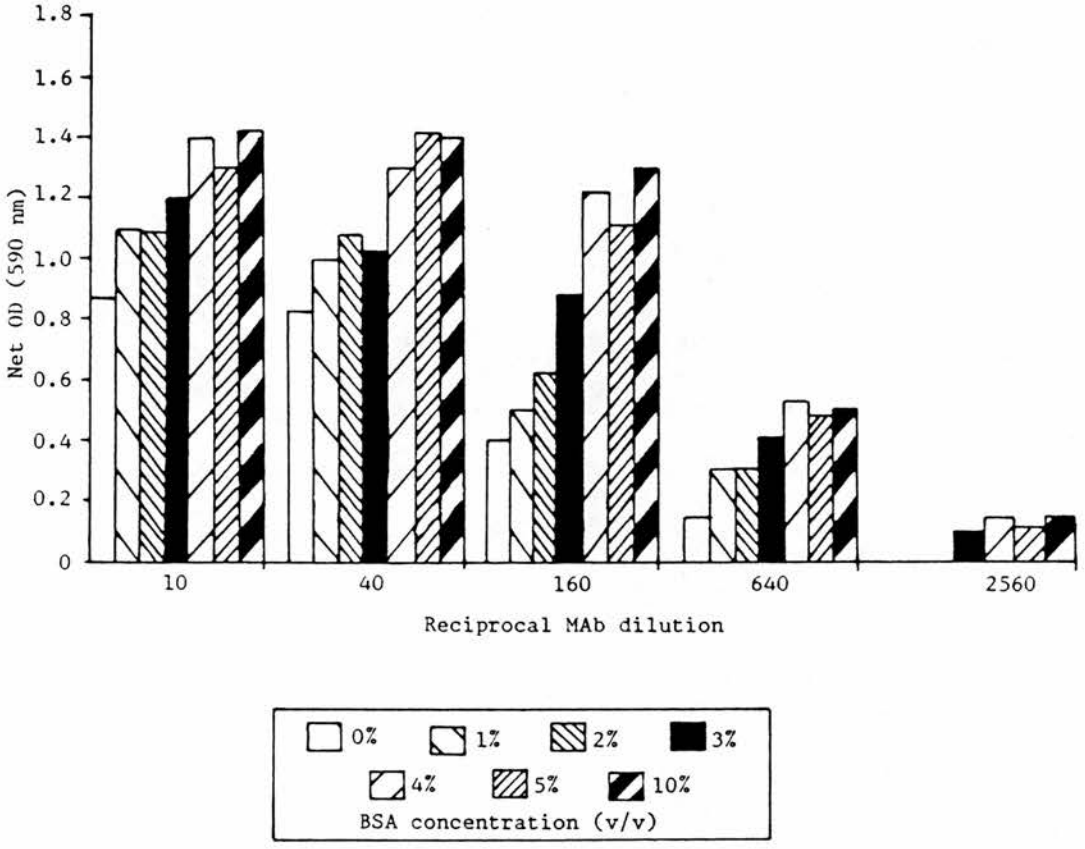


Figure 28. ELISA activity of anti-core specific MAb 27.150.3 against proteinase K extracted *E. coli* R1 LPS, postcoated with 0-10% v/v bovine serum albumin (BSA). Each histogram bar represents the mean optical density value of triplicate readings.

Optimization of post-coating concentrations of BSA

Microplates coated with 1:10 dilutions of R1 LPS-polymyxin complex were post-coated with varying concentrations of BSA in PBS (1%, 2%, 3%, 4%, 5%, 10% w/v). Cross-reactive, anti-core specific MAb 27.150.3 was titrated against each of the R1 coated plates, post-coated with the different BSA concentrations (Figure 28). Each MAb dilution showed increasing OD with increasing BSA post-coat concentration up to 4%, above which there was no significant increase. Since a 5% w/v BSA concentration had been used for all other ELISA coating procedures this was also used for post-coating microplates coated with proteinase K extracted LPS.

Reproducibility of proteinase K extracted LPS-polymyxin complex coating and LPS coating alone

Proteinase K extracted LPS-polymyxin complexes and uncomplexed LPS from both rough and smooth bacterial strains were each coated to microplates on three separate occasions to assess the reproducibility of the coating procedure in ELISA (Figure 29). Mean ELISA OD values for *E. coli* O18-serotype specific MAb 184.2.5.5 against the corresponding S-LPS were similar at each MAb dilution for both complexed and uncomplexed LPS (Figure 29a and b). However, the reproducibility of these OD values as measured by standard deviation (SD) was greater for microplates coated with complexed LPS at each MAb dilution. Microplates coated with R-LPS-polymyxin complexes and probed with anti-core MAb 27.150.3 had higher mean OD values and lower SD values at each MAb dilution than microplates coated with LPS alone (Figure 29c and d). The SD of both complexed and uncomplexed R-LPS preparations were higher than equivalent S-LPS preparations.

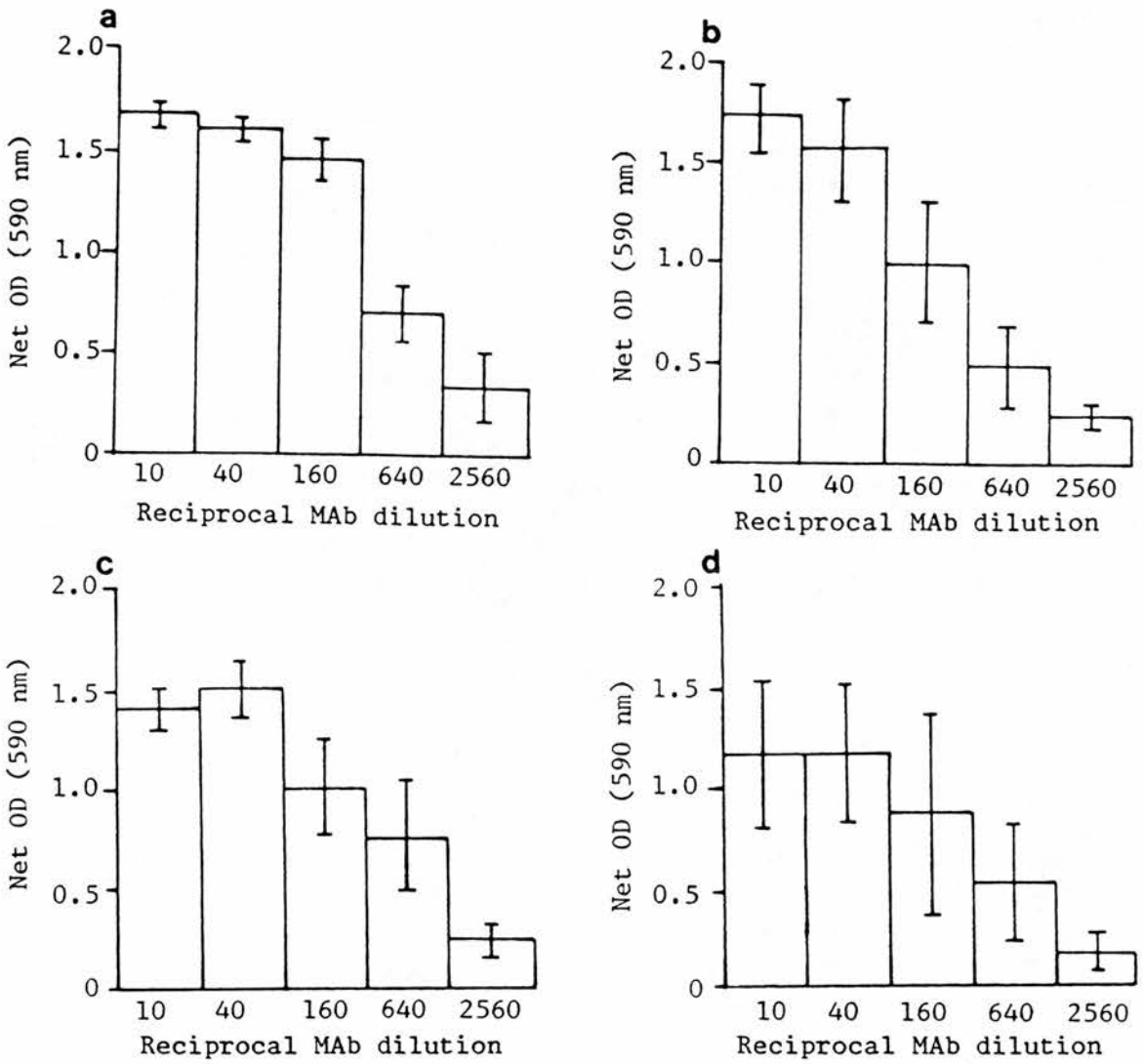


Figure 29. Comparison of ELISA activity of microplates coated with proteinase K extracted LPS complexed with either polymyxin (a & c) or no polymyxin (b & d) and probed with an anti-LPS MAb.
a & b represent MAb 184.2.5.5 against *E. coli* O18:K1 (S-LPS)
c & d represent MAb 27.150.3 against *E. coli* R1 (R-LPS)
Bars represent the SD of triplicate experiments on microplates coated on 3 separate occasions.

Inhibition of ELISA reactivity of anti-LPS monoclonal antibodies by five different LPS preparations

Whole bacterial cells, purified LPS (complexed and uncomplexed) and proteinase K extracted LPS (complexed and uncomplexed) were compared for their ability to inhibit the ELISA reactivity of anti-LPS MAbs. Purified LPS was prepared in a doubling dilution series from an original concentration of 0.1 mM for use as an inhibitor. Doubling dilutions of proteinase K extracted LPS were prepared from an approximate LPS concentration of 0.1 mM. Whole cells were prepared in a ten-fold dilution series from $1x10^9$ cells ml^{-1}. All inhibitor dilutions were mixed with an equal volume of MAb (diluted 1:40 in dilution buffer) and preincubated at 37°C for 30 min before adding to ELISA.

Figure 30a and b represent ELISA inhibition by five LPS containing preparations of O-antigen specific MAb 184.2.5.5 against microplates coated with either purified LPS-polymyxin complex (a) or proteinase K extracted LPS-polymyxin complex (b). Figure 31a and b represent ELISA inhibition by five LPS containing preparations of core-specific MAb 27.150.3 against microplates coated as above for (a) and (b). Inhibition of ELISA reactivity occurred with all LPS preparations, residual ELISA activity being inversely proportional to inhibitor concentration in most cases. In general, the weakest inhibitor of ELISA reactivity was the whole cell preparation, whilst the most efficient inhibitor was the purified LPS-polymyxin complex. The inhibitory activities of proteinase K extracted LPS preparations were comparable to the corresponding purified LPS preparations. Residual ELISA reactivity of MAb 184.2.5.5 (Figure 30) after inhibition with either purified or proteinase K extracted LPS (complexed or uncomplexed) was higher against coated purified LPS (a) than proteinase K extracted LPS (b).

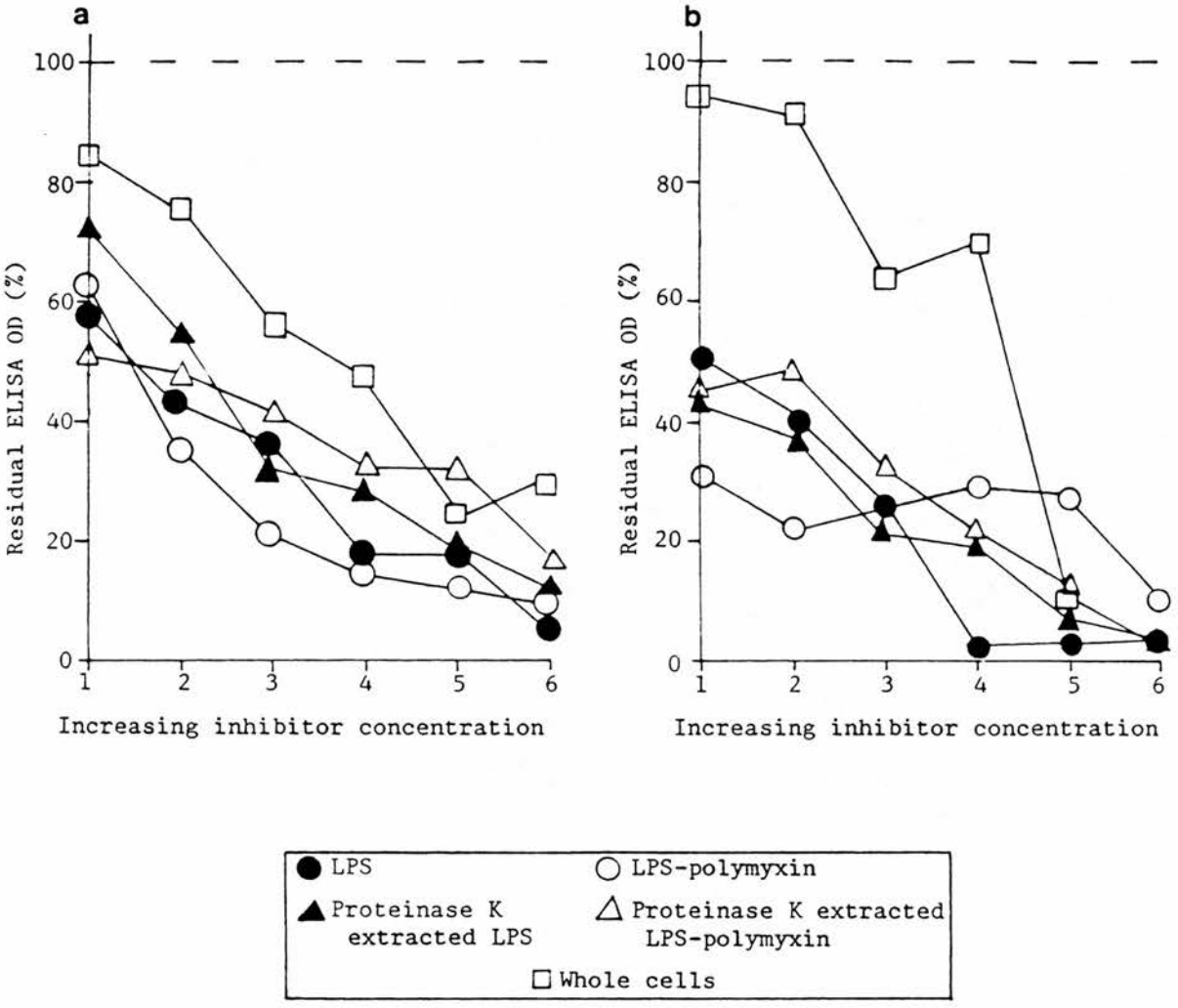


Figure 30. Inhibition of ELISA reactivity of O18, O-antigen specific MAb 184.2.5.5 (diluted 1:40) against purified *E. coli* O18 LPS (a) and proteinase K extracted *E. coli* O18 LPS (b) with: whole cells (ten-fold dilution series from $1x10^{9}$ cells ml^{-1}); purified LPS or purified LPS complexed with polymyxin (doubling dilutions from an original concentration of 0.1 mM), and proteinase K extracted LPS or proteinase K extracted LPS complexed with polymyxin (doubling dilutions from an original concentration of approximately 0.1 mM). Residual ELISA reactivity in the presence of inhibitor is expressed as a percentage of reactivity with no inhibitor (the 100% value, represented by the dotted line).

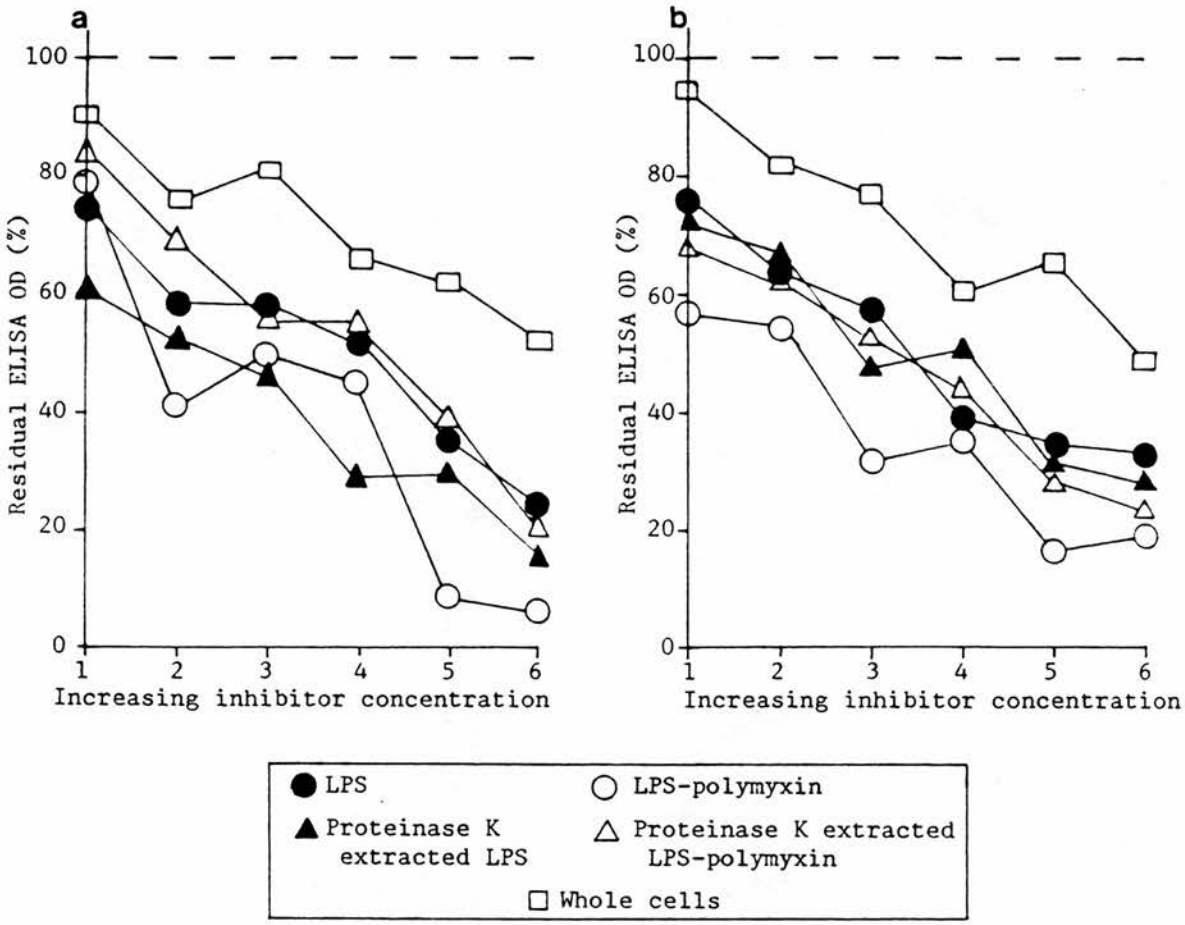


Figure 31. Inhibition of ELISA reactivity of core-specific MAb 27.150.3 (diluted 1:40) against purified *E. coli* R1 LPS (a) and proteinase K extracted *E. coli* R1 LPS (b) with: whole cells (ten-fold dilution series from $1x10^{9}$ cells ml^{-1}; purified LPS or purified LPS complexed with polymyxin (doubling dilutions from an original concentration of 0.1 mM) and proteinase K extracted LPS or proteinase K extracted LPS complexed with polymyxin (doubling dilutions from an original concentration of approximately 0.1 mM). Residual ELISA reactivity in the presence of inhibitor is expressed as a percentage of reactivity with no inhibitor (the 100% value, represented by the dotted line).

The equivalent comparison for MAb 27.150.3 (Figure 31) showed that residual ELISA reactivity was similar for microplates coated with purified LPS (a) or proteinase K extracted LPS (b).

1.6 BINDING OF ANTI-LIPOPOLYSACCHARIDE MONOCLONAL ANTIBODIES AGAINST PROTEINASE K EXTRACTED LIPOPOLYSACCHARIDE IN ELISA

The ELISA binding activities of MAbs against proteinase K whole cell digests of equivalent bacterial antigens used in the heat-killed bacterial ELISA are shown in Figures 32-40. Optical density readings for MAbs against the different LPS samples tended to be higher compared to the responses of the MAbs with heat-killed cell antigens. This may reflect differences in the amount of LPS antigen added to the wells of the ELISA microplates, the coating efficiency of the different preparations or the presentation of the LPS antigens.

The reactivity patterns of individual MAbs against the proteinase K extracted LPS preparations reflected results of the heat-killed ELISA (Figures 6-16), rather than those of immunoblotting studies (Figures 17-22). This was highlighted by MAbs 43.11.5.1 (Figure 38) and 43.27.11.2 (Figure 39) which cross-reacted against *E. coli* R- and S-LPS antigens in ELISA, and reacted almost exclusively with the *E. coli* R1 core in immunoblots. MAbs 184.2.5.5 and 185.1.2.2 again demonstrated specificity for *E. coli* serotypes O18 and O6 respectively (data not shown).

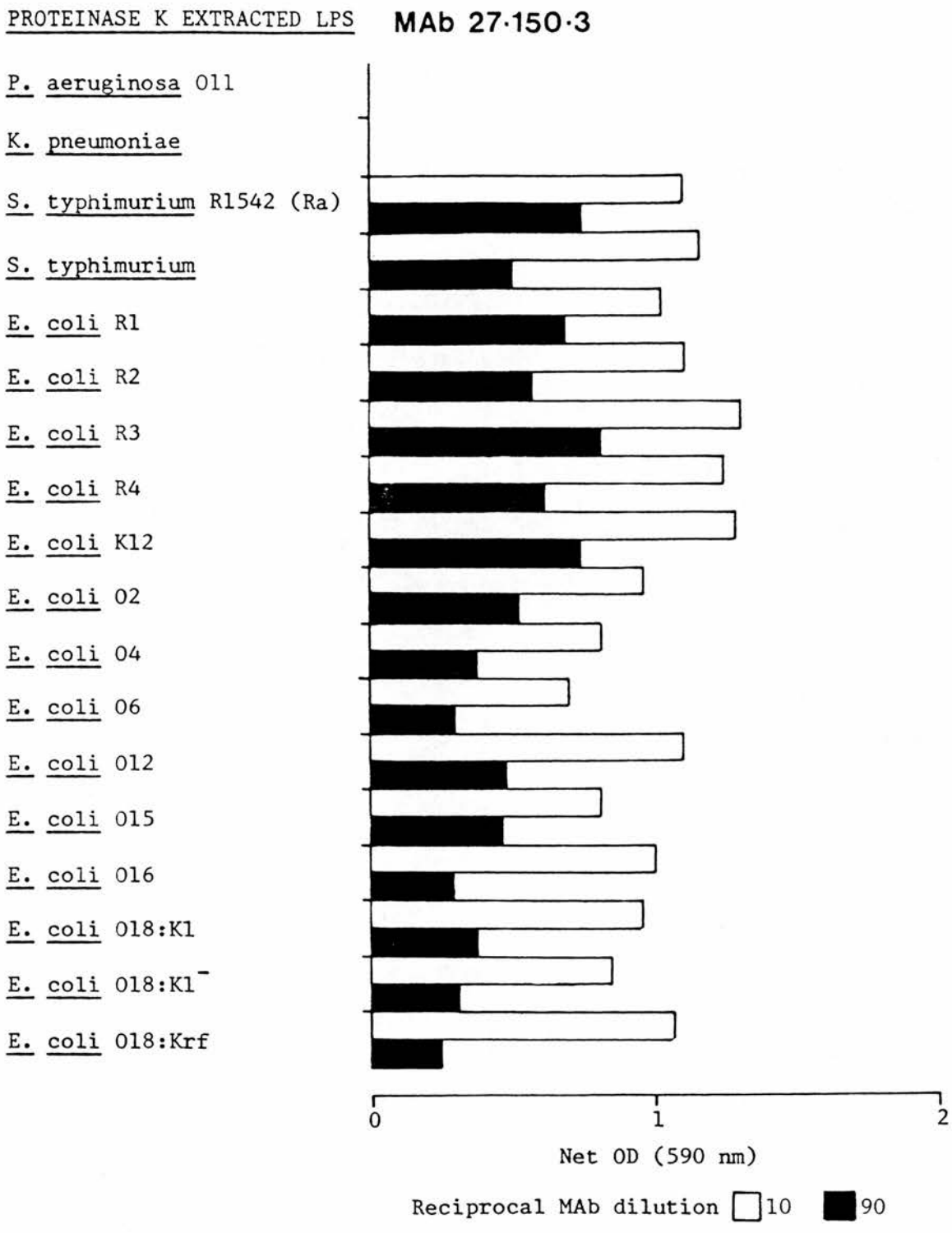


Figure 32. ELISA activity of MAb 27.150.3 against proteinase K whole cell digests of *E. coli* and non-*E. coli* rough and smooth cell types.

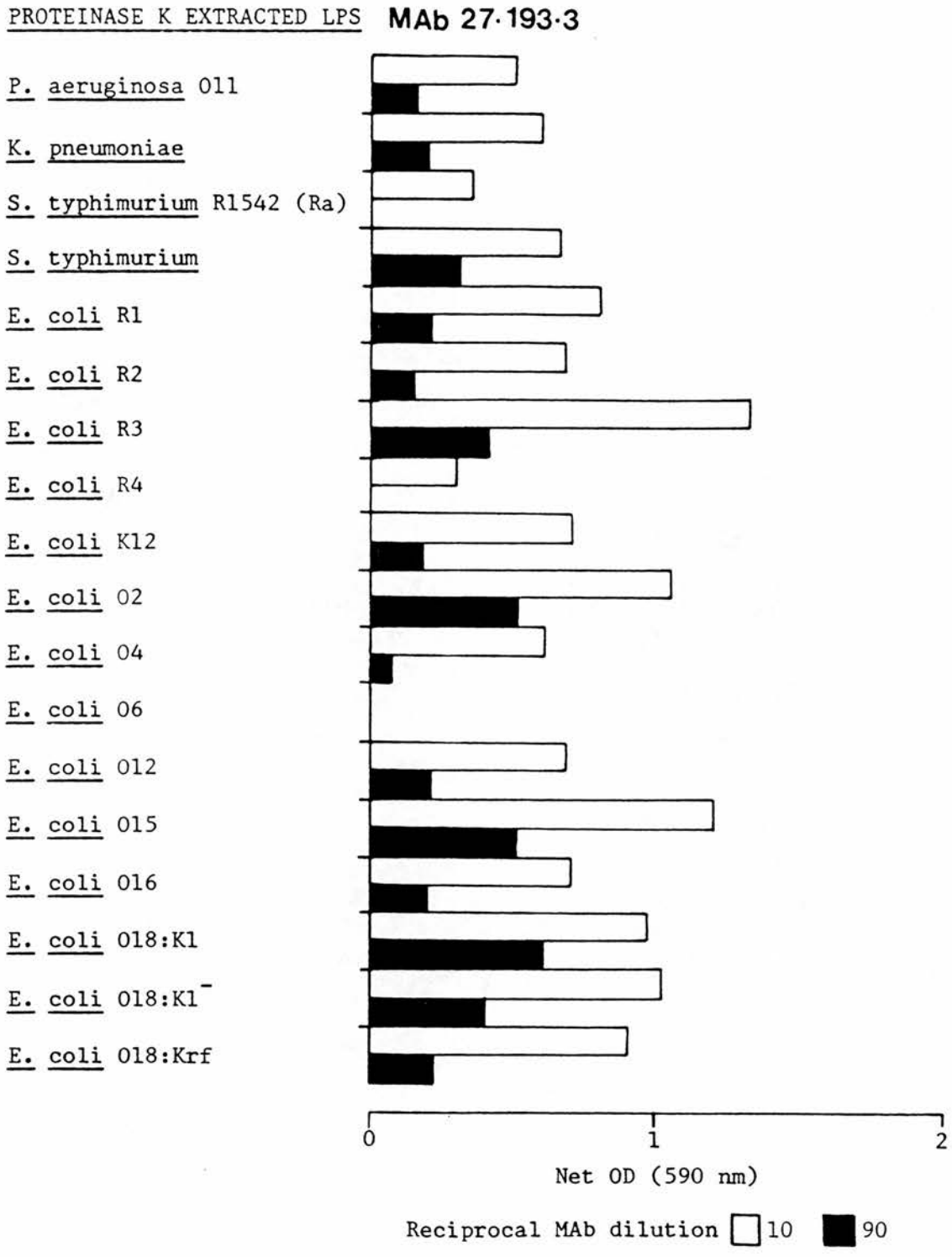


Figure 33. ELISA activity of MAb 27.193.3 against proteinase K whole cell digests of *E. coli* and non-*E. coli* rough and smooth cell types.

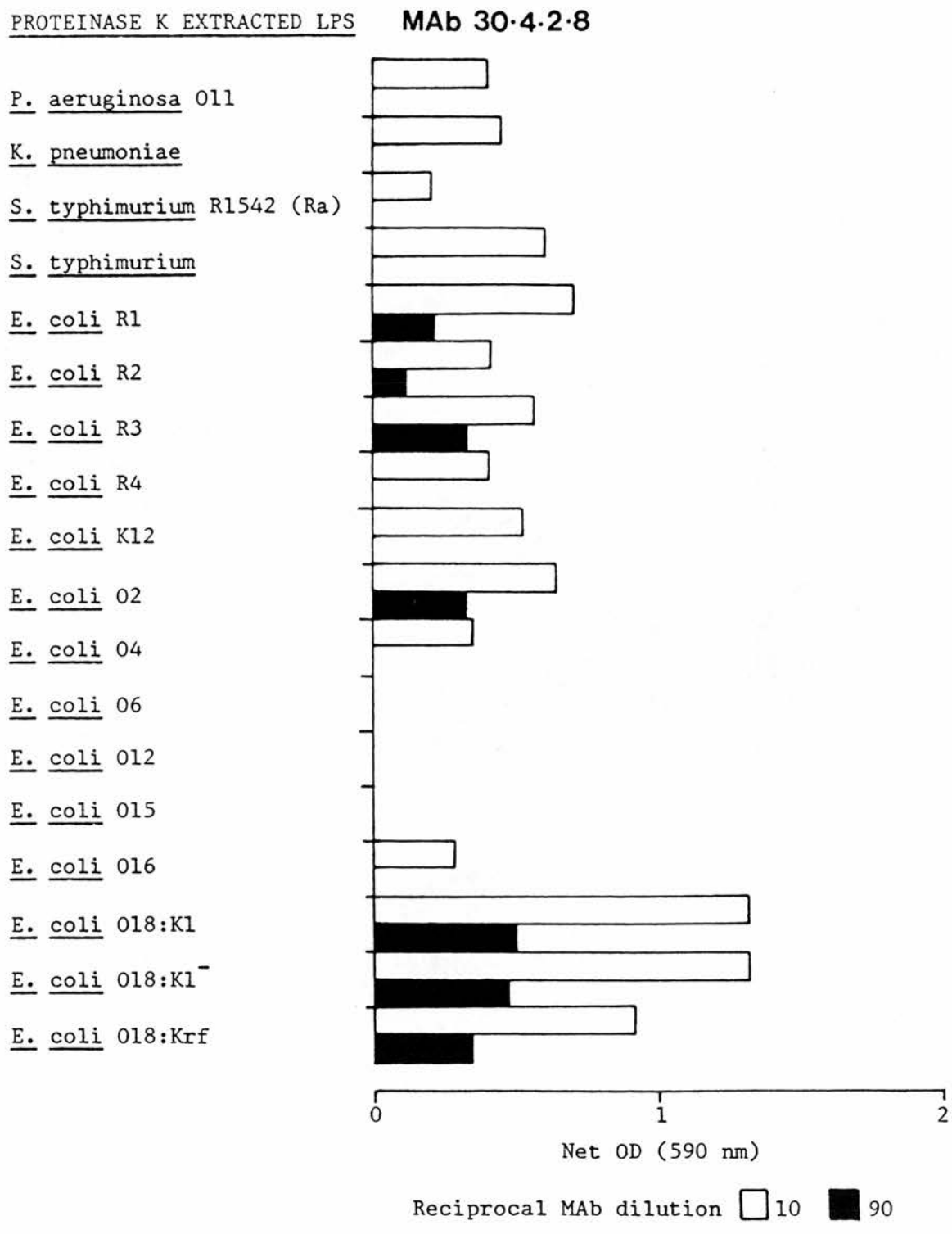


Figure 34. ELISA activity of MAb 30.4.2.8 against proteinase K whole cell digests of *E. coli* and non-*E. coli* rough and smooth cell types.

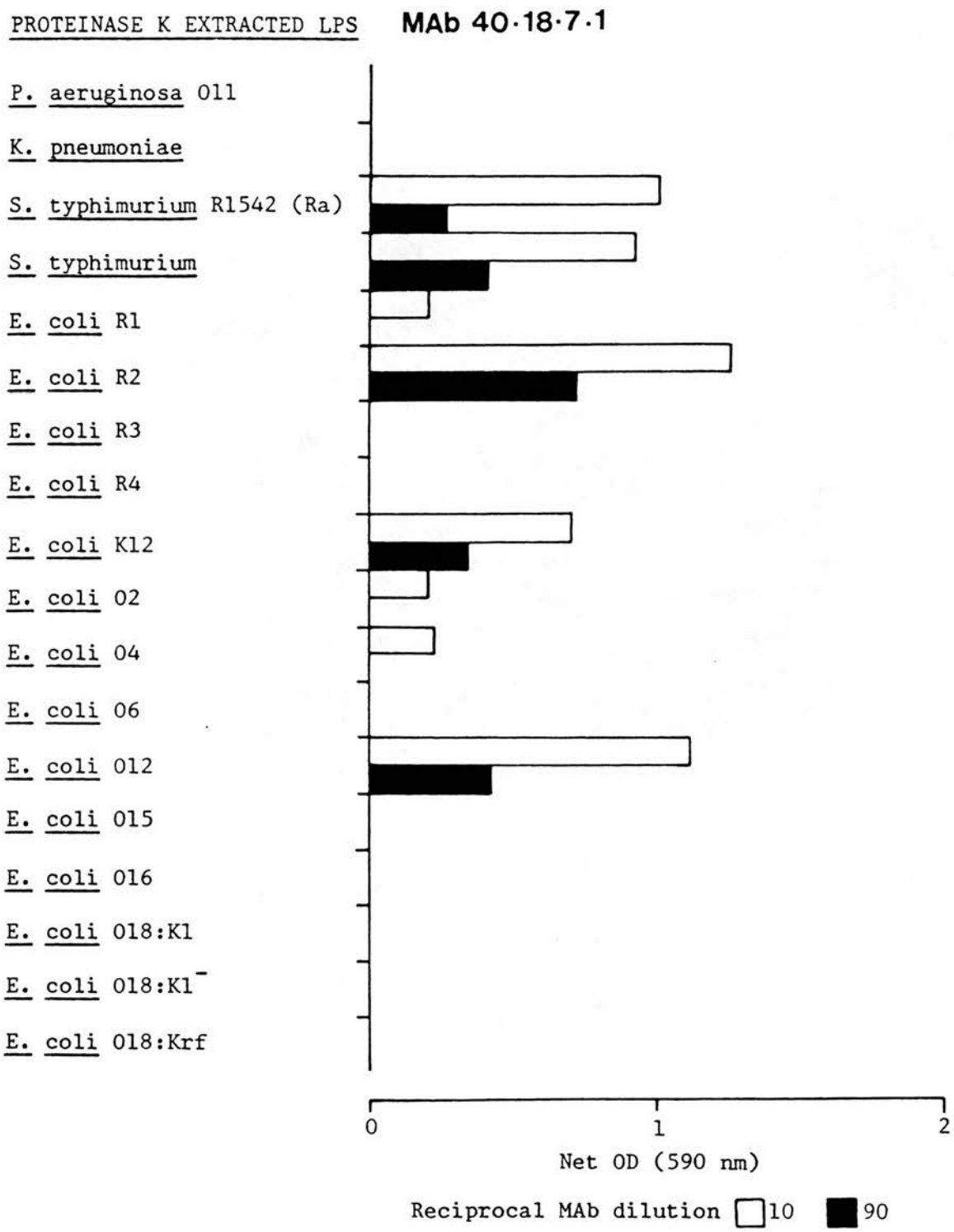


Figure 35. ELISA activity of MAb 40.18.7.1 against proteinase K whole cell digests of *E. coli* and non-*E. coli* rough and smooth cell types.

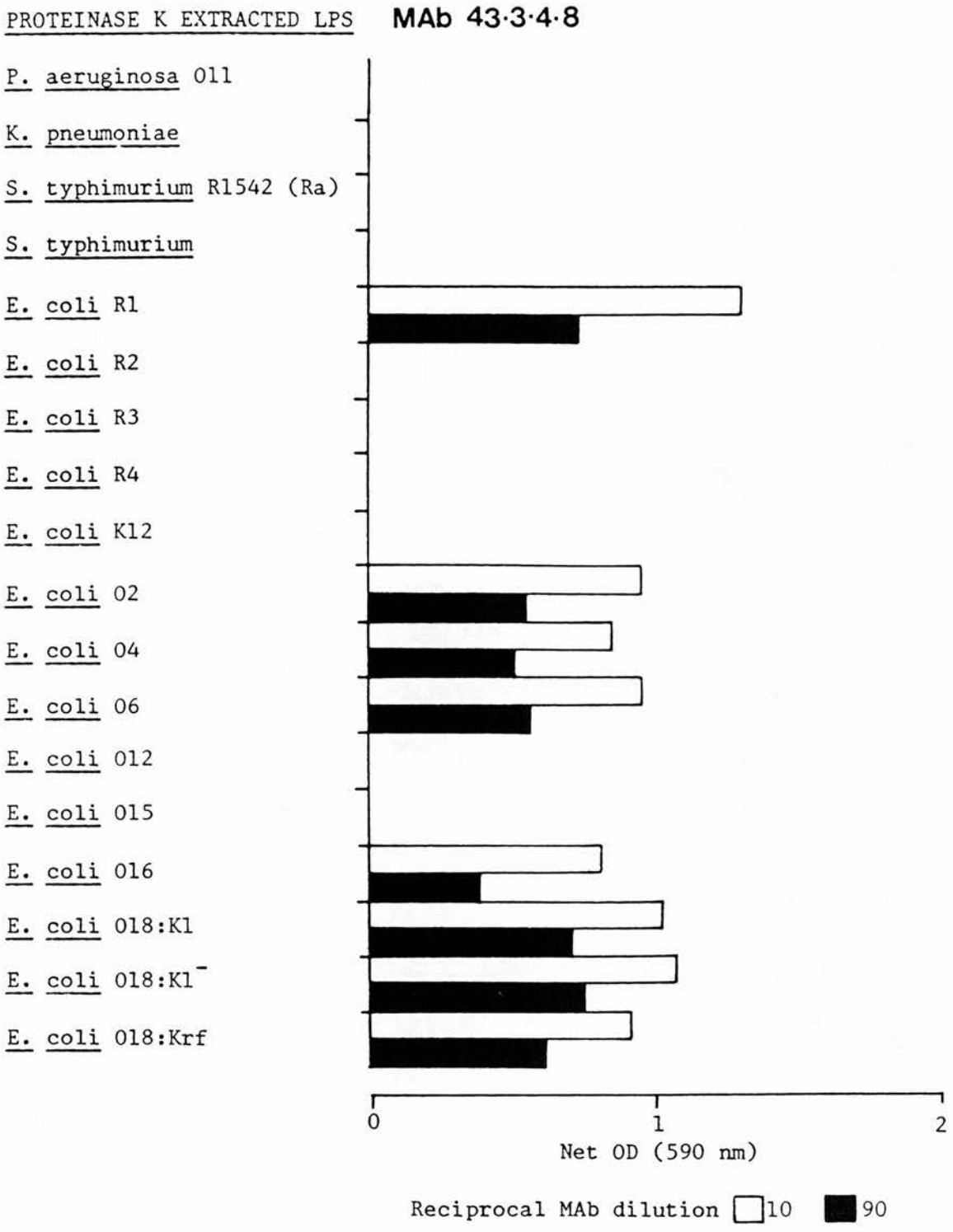


Figure 36. ELISA activity of MAb 43.3.4.8 against proteinase K whole cell digests of *E. coli* and non-*E. coli* rough and smooth cell types.

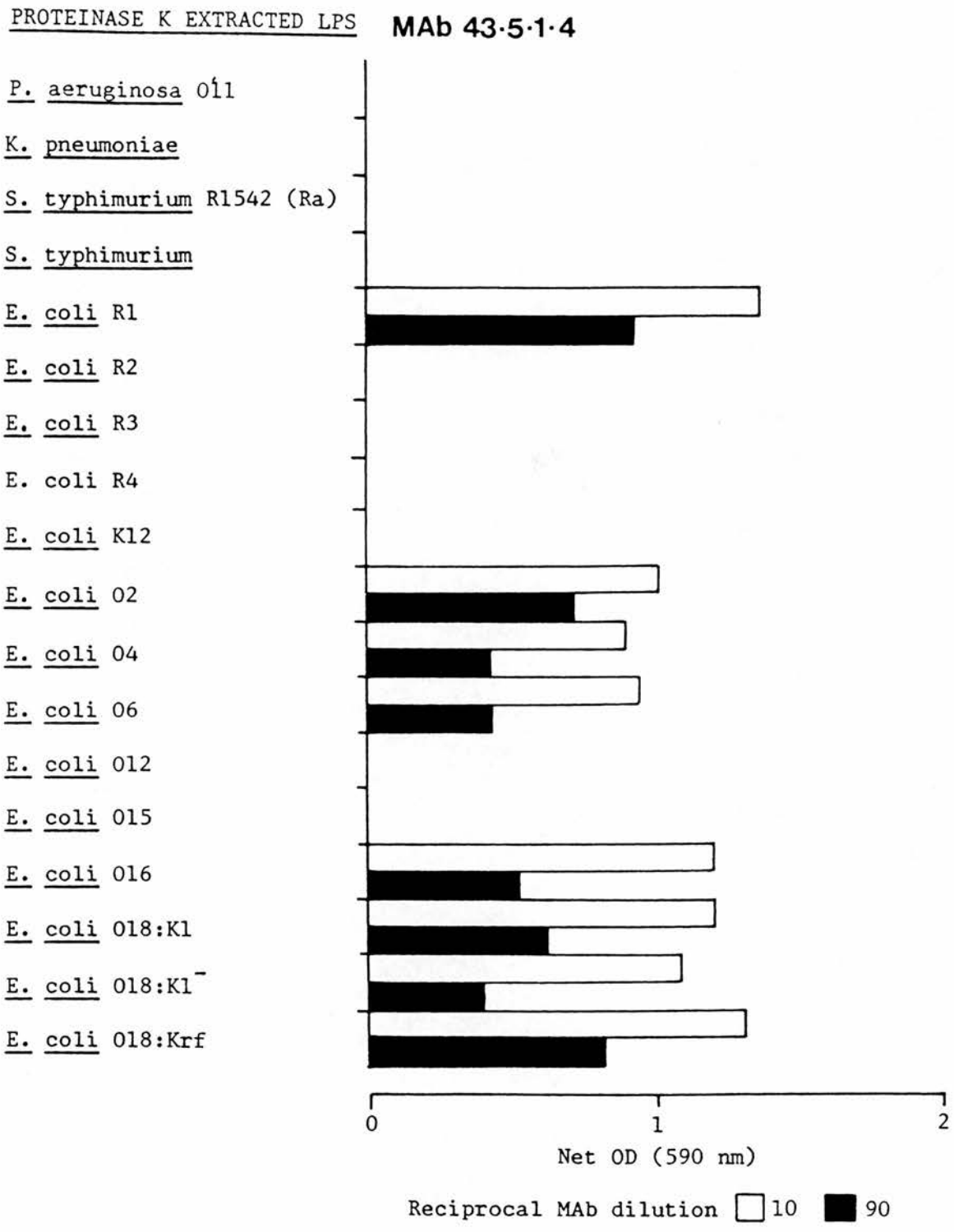


Figure 37. ELISA activity of MAb 43.5.1.4 against proteinase K whole cell digests of *E. coli* and non-*E. coli* rough and smooth cell types.

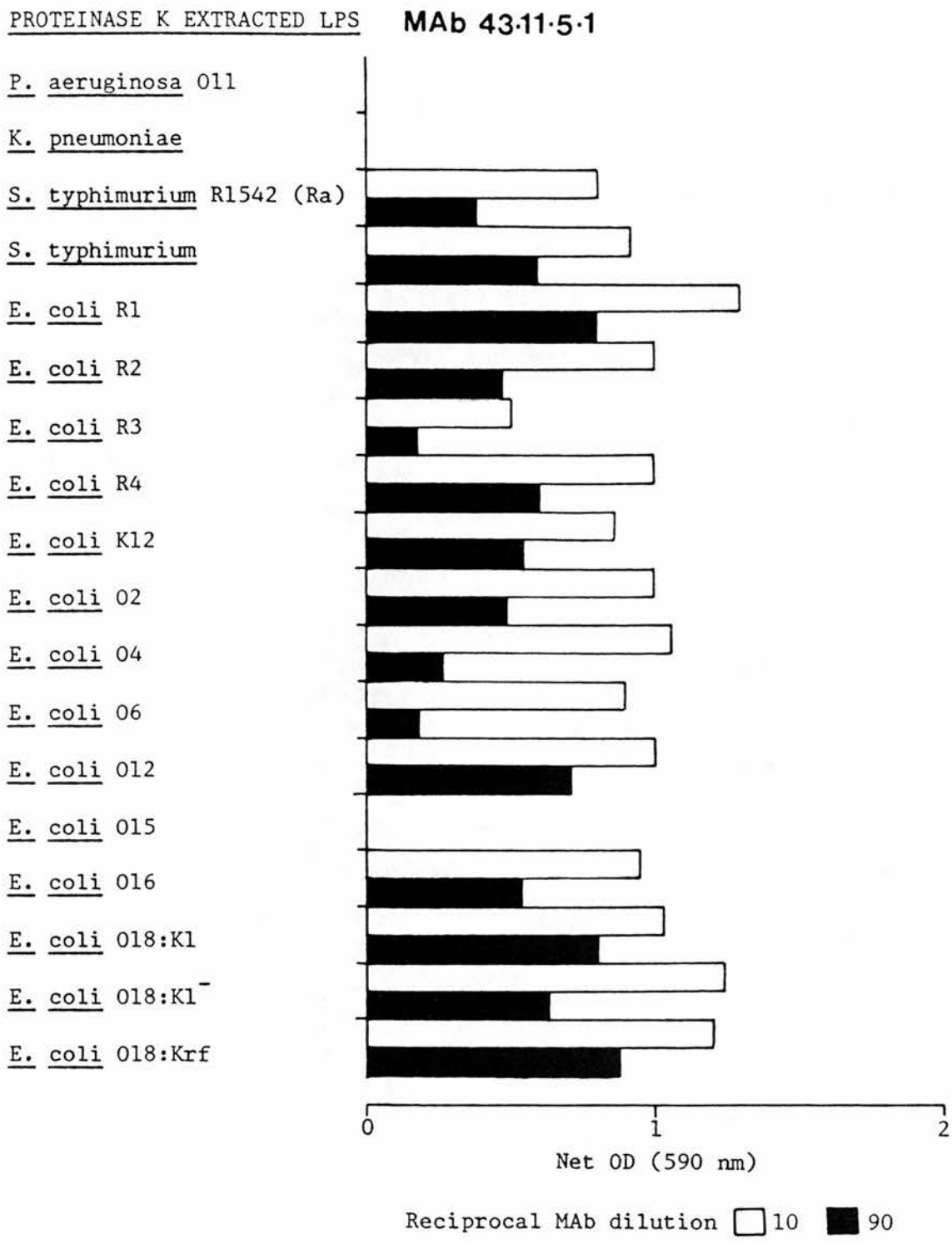


Figure 38. ELISA activity of MAb 43.11.5.1 against proteinase K whole cell digests of *E. coli* and non-*E. coli* rough and smooth cell types.

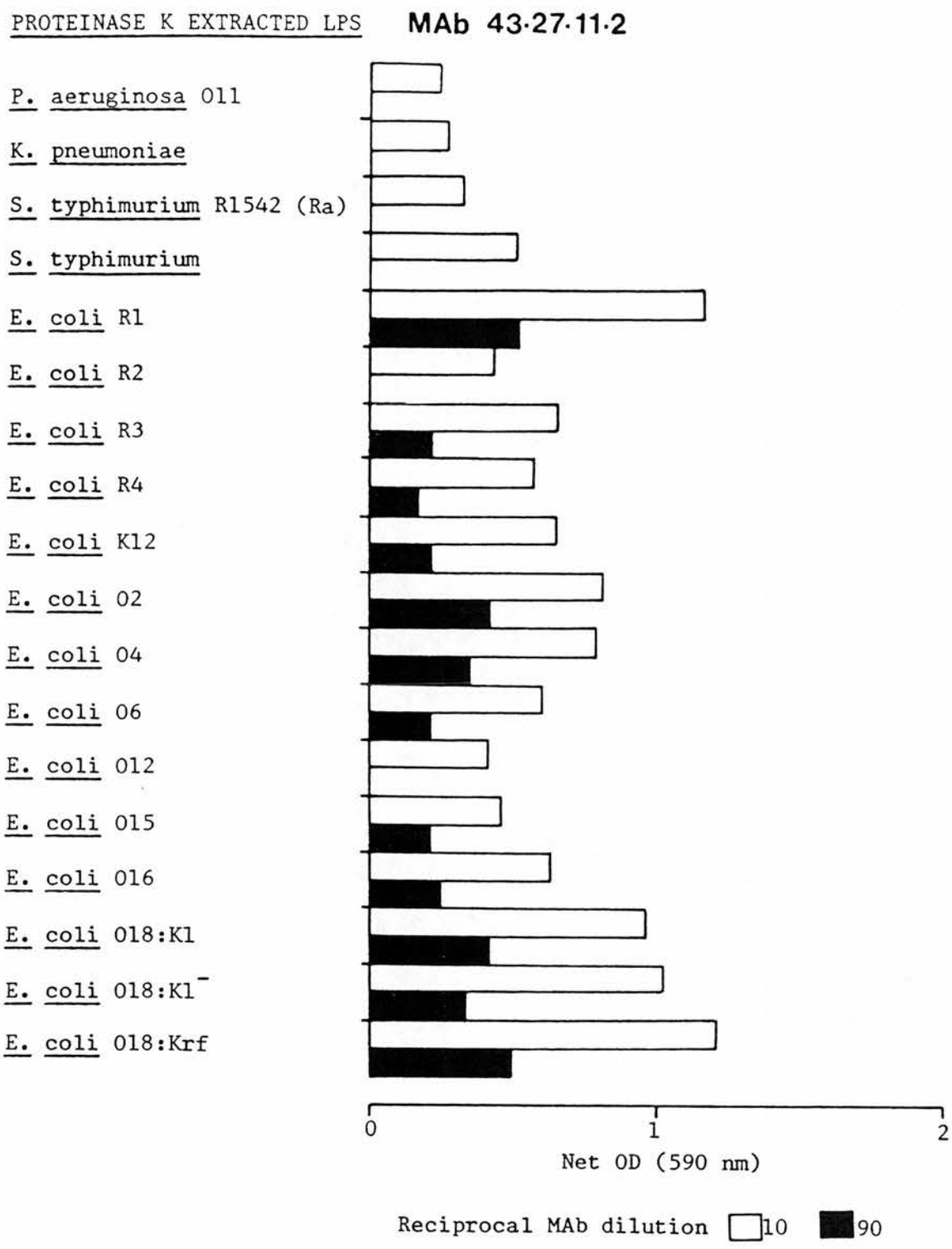


Figure 39. ELISA activity of MAb 43.27.11.2 against proteinase K whole cell digests of *E. coli* and non-*E. coli* rough and smooth cell types.

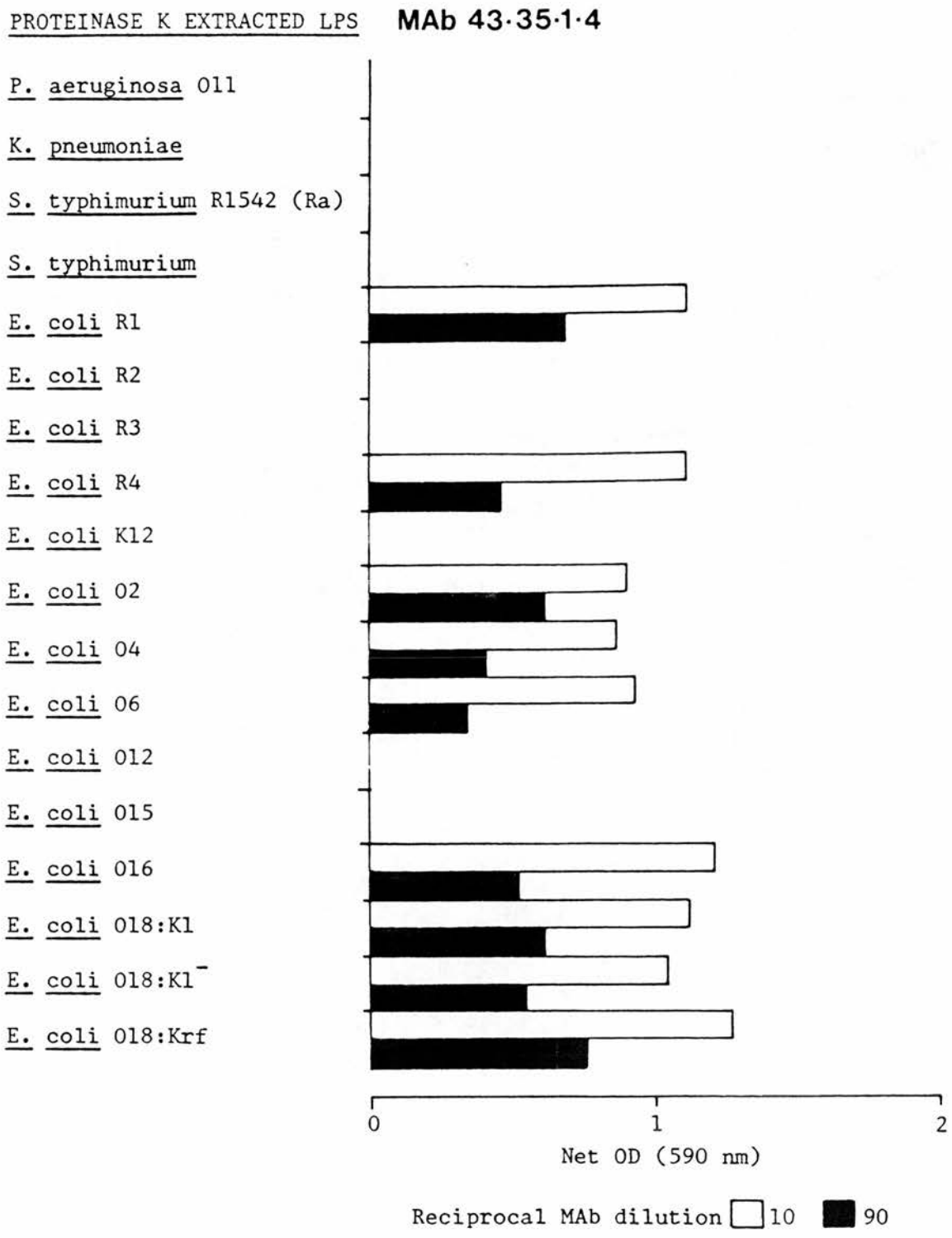


Figure 40. ELISA activity of MAb 43.35.1.4 against proteinase K whole cell digests of *E. coli* and non-*E. coli* rough and smooth cell types.

CHAPTER 2

DEVELOPMENT OF A CAPTURE ELISA FOR THE DETECTION OF ESCHERICHIA COLI LIPOPOLYSACCHARIDE IN SOLUTION

The aim of this study was to use MAbs in a capture ELISA system for the detection of *E. coli* LPS in solution. Several MAbs were selected (on the basis of their specificities as illustrated in Chapter 1) for the detection of *E. coli* core types R1-R4, specific *E. coli* core types of either R1 or R3, and *E. coli* 018 O-antigen. The assays were developed with the ultimate aim of detecting LPS in the serum of septic patients.

Selected MAbs included:

MAb	Preferential Specificity	Isotype
27.150.3	*E. coli* core cross-reactive	IgG2a
27.193.3	*E. coli* R3 core	IgM
30.4.2.8	*E. coli* 018 O-antigen	IgM
40.18.7.1	*E. coli* R2 core	IgG3
43.3.4.8	*E. coli* R1 core	IgG3
43.11.5.1	*E. coli* core cross-reactive	IgG2a/IgM
43.27.11.2	*E. coli* core cross-reactive	IgG3

2.1 PURIFICATION OF MONOCLONAL ANTIBODY PREPARATIONS

Bulk growth supernates of selected MAbs were concentrated twenty-fold using a tangential-flow filtration system. The presence of antibody in the retentate and absence in the filtrate was checked by ELISA. The immunoglobulins in each concentrate were further purified by ammonium sulphate precipitation. The presence of antibody in the final preparation was confirmed by titrating in ELISA. Equal volumes of MAb preparations before and after ammonium sulphate precipitation were analysed by PAGE, and stained with Coomassie blue as shown in Figure

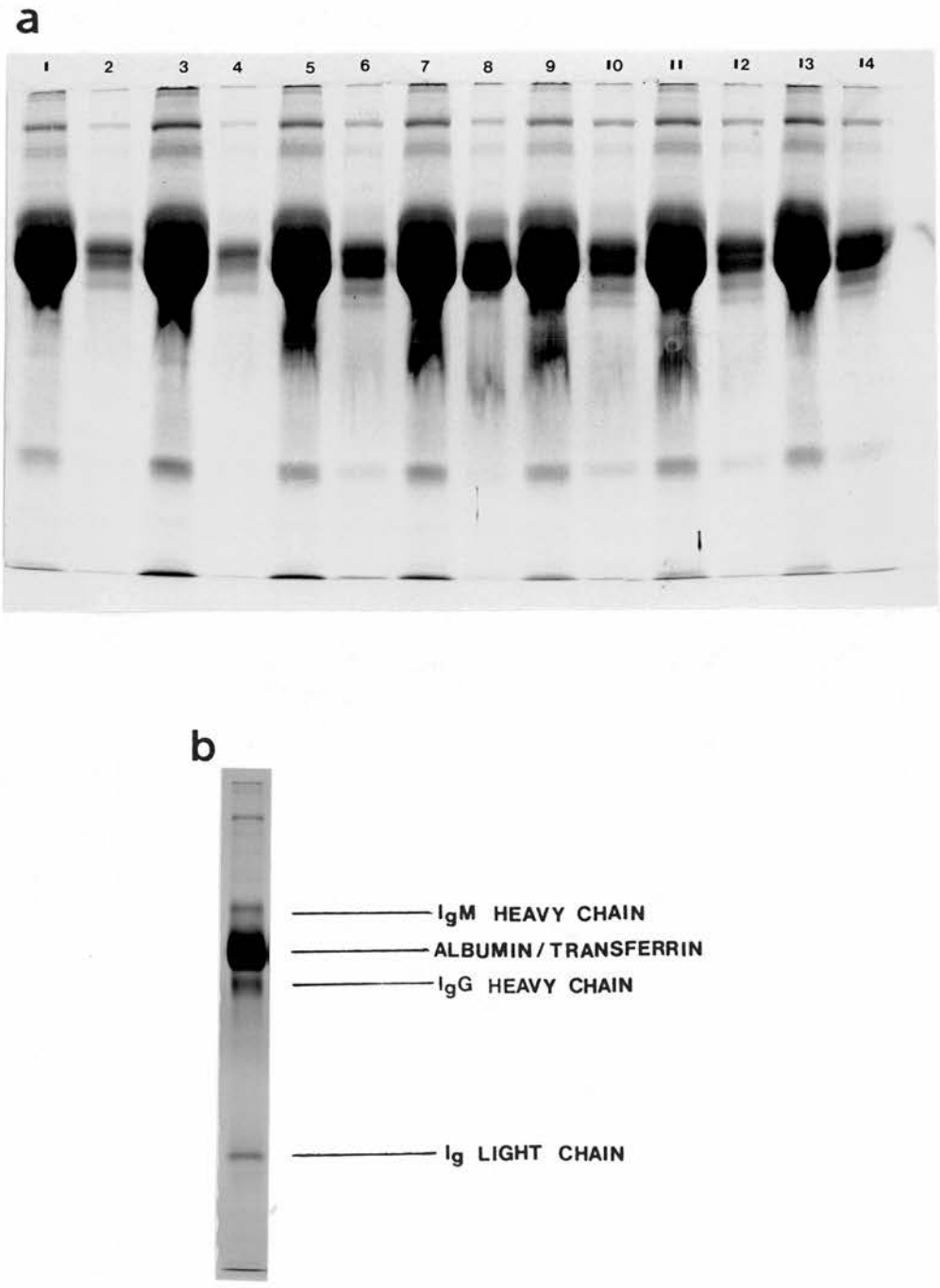


Figure 41. a) Coomassie blue stained profiles of proteins found in concentrated supernatants containing MAb before and after ammonium sulphate precipitation, separated by SDS-PAGE (12% w/v acrylamide). Track 1, 27.150.3 (before); Track 2, 27.150.3 (after); Track 3, 40.18.7.1 (before); Track 4, 40.18.7.1 (after); Track 5, 43.3.4.8 (before); Track 6, 43.3.4.8 (after); Track 7, 43.11.5.1 (before); Track 8, 43.11.5.1 (after); Track 9, 43.27.11.2 (before); Track 10, 43.27.11.2 (after); Track 11, 27.193.3 (before); Track 12, 27.193.3 (after); Track 13, 30.4.2.8 (before); Track 14, 30.4.2.8 (after). b) Silver stained profiles of proteins found in human serum.

41a. Ammonium sulphate precipitation of the MAb preparations resulted in a substantial reduction in the number of contaminating proteins including albumin and transferrin. Bands representing MAb components were relatively weak compared to bands obtained for more abundant immunoglobulins found in normal human serum (Figure 41b).

Ammonium sulphate precipitated MAbs were further purified by either protein-A affinity chromatography (IgG) or DEAE ion exchange chromatography (IgM). Since 'MAb' 43.11.5.1 contained both IgG2a and IgM isotypes, it appears that this MAb is actually the product of two hybridomas. The IgG2a was purified and separated from the IgM by protein-A chromatography and the IgM purified by DEAE affinity chromatography. The reactivity of each MAb was checked in ELISA. The specificity of the IgG component was shown to be identical to the original concentrated supernate preparation and used for all subsequent detection studies. The specificity of the IgM component was similar to the concentrated supernate preparation, although it showed weaker reactivity against individual LPS preparations.

Purified MAb samples (prepared for electrophoresis in the presence of 2-mercaptoethanol) were separated by SDS-PAGE and stained with Coomassie blue as shown in Figure 42. Molecular masses of separated heavy and light chains of IgG and IgM antibodies were estimated from a calibration curve of log molecular mass of protein standards against Rf (the distance moved by a standard protein through the separating gel, divided by the distance moved by bromophenol blue marker). The light chains of IgG and IgM antibodies had a calculated molecular mass of 23,000, whilst the heavy chains had calculated molecular masses of 52,000 and 71,000 for IgG and IgM antibodies respectively. These

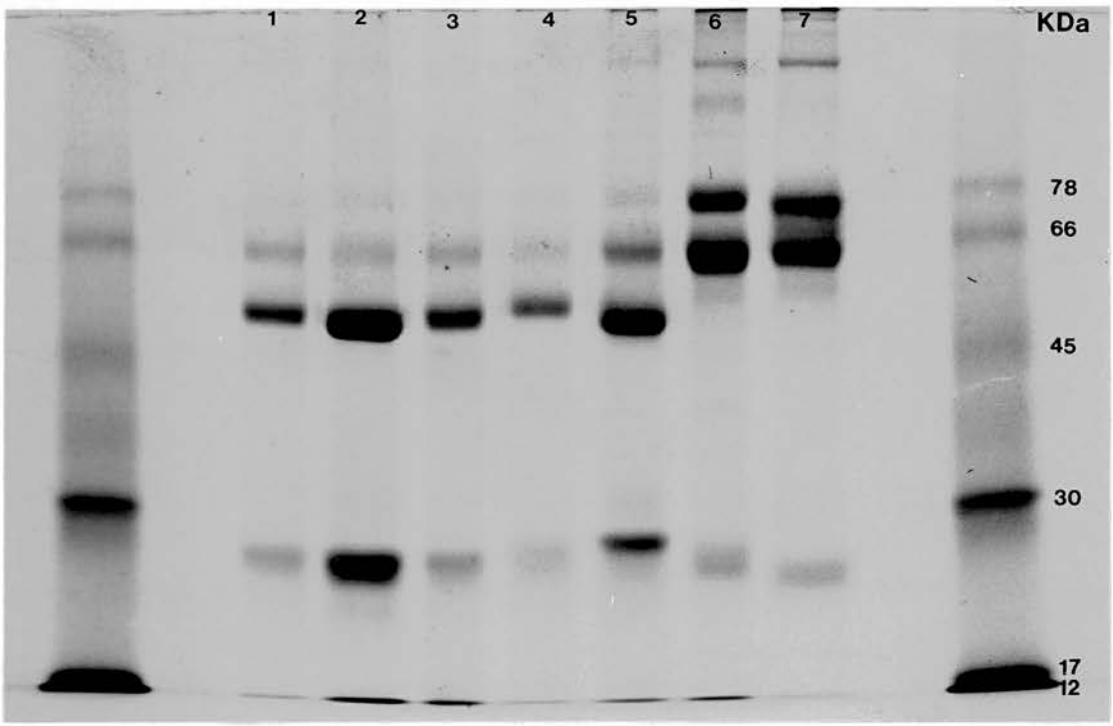


Figure 42. Coomassie blue stained profiles of purified MAb preparations separated by SDS-PAGE (12% w/v acrylamide). Track 1, 27.150.3 (IgG2a); Track 2, 40.18.7.1 (IgG3); Track 3, 43.3.4.8 (IgG3); Track 4, 43.11.5.1 (IgG2a); Track 5, 43.27.11.2 (IgG3); Track 6, 27.193.3 (IgM); Track 7, 30.4.2.8 (IgM). Molecular masses of protein standards are indicated.

values were similar to those documented by Parham (1986). All purified MAb preparations still contained a non-MAb protein of approximately 66,000 molecular mass. Trace amounts of this protein were present in IgG preparations as revealed by staining intensity, whilst an equal amount to the heavy chain of IgM antibodies was present in IgM preparations.

2.2 BIOTIN LABELLING OF MONOCLONAL ANTIBODIES

The degree of biotinylation of mouse MAbs yielding the highest reactivity was determined by reacting various ratios of biotin to free amino groups of MAbs. The different ratios of biotinylated MAbs were tested in ELISA against individual cocktails of *E. coli* R- and S-LPS. The results of representative IgG and IgM MAbs are illustrated in Figure 43. All OD readings were expressed after subtraction of the relevant background control.

Conjugation of MAb 43.3.4.8 and 27.193.3 with 1:1 and 2:1 biotin: MAb/amino acid ratios appeared to produce the most usable reagents in each case. The 4:1 ratio gave the lowest OD readings for each dilution of MAb, whilst the 1:1 ratio gave the maximal. As the dilution of each biotinylated MAb increased, OD readings for wells with R-LPS were usually higher than those obtained for wells coated with S-LPS. Since both MAbs recognize unsubstituted core-glycolipid LPS, the increased sensitivity with R-LPS may be due to the presence of more available core LPS than in S-LPS. The masking effect of O-antigen side chains is also likely to influence epitope recognition. The equivalent results for other MAbs gave similar results to those presented for 43.3.4.8 and 27.193.3.

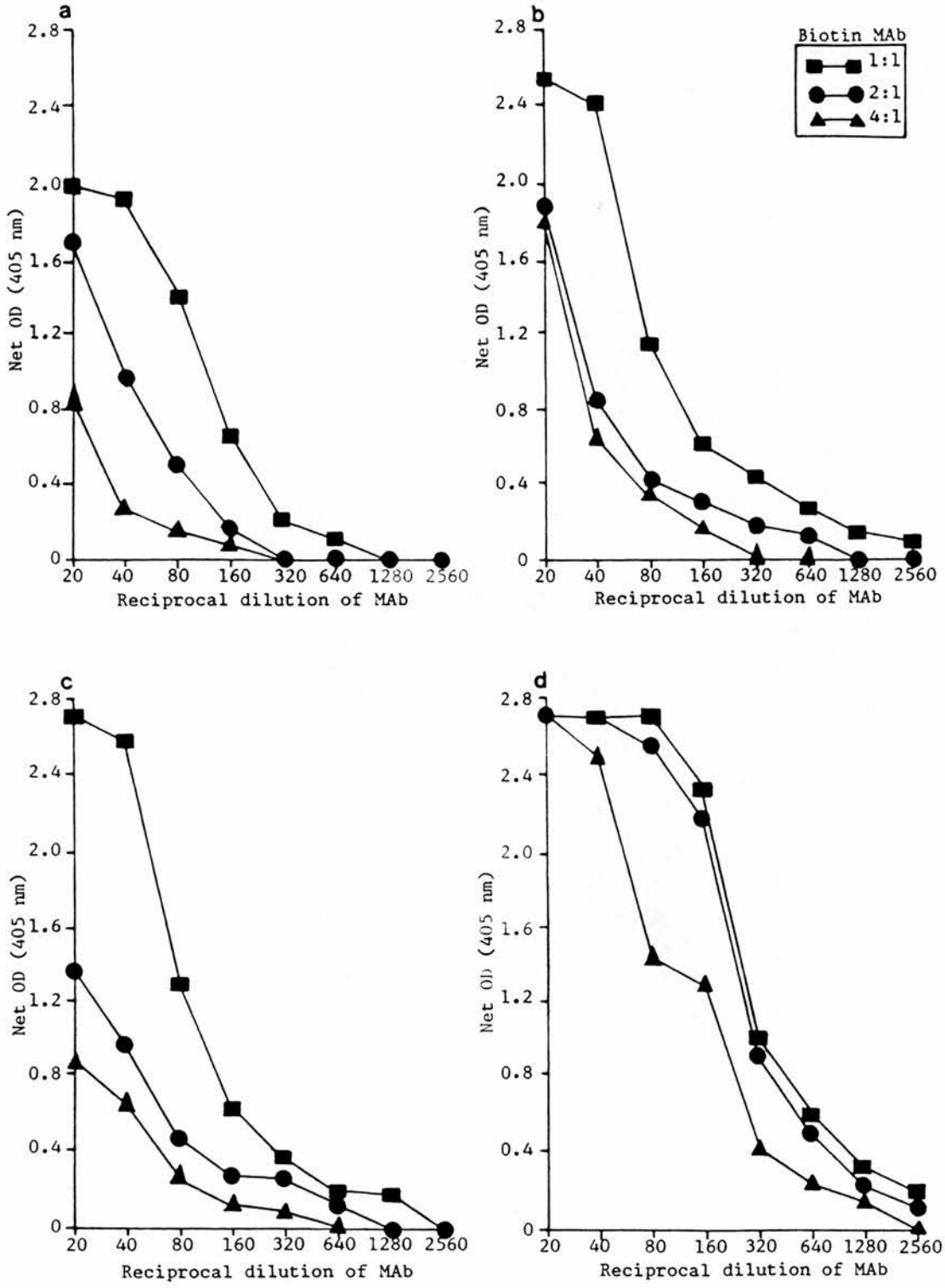


Figure 43. Reactivity in ELISA of MAbs 43.3.4.8 (a & b) and 27.193.3 (c & d) coupled with different ratios of biotin. Reactions against a cocktail of smooth (a & c) and rough (b & d) *E. coli* LPS were measured. The OD values obtained with streptavidin-alkaline phosphatase (SAP) were measured at 405 nm.

2.3 BIOTIN-STREPTAVIDIN AMPLIFICATION ELISA SYSTEMS

The sensitivity of two different conjugate systems for bio-MAbs against *E. coli* S-LPS and R-LPS were compared. The conjugate systems included: i) alkaline phosphatase biotin-streptavidin (the amplification system) and ii) alkaline phosphatase labelled streptavidin.

The results obtained for representative bio-MAbs (1:1) 43.3.4.8 and 27.193.3 with the different conjugate systems are shown in Figure 44. The conjugate system employing alkaline phosphatase labelled biotin-streptavidin complex produced the highest OD values for each respective dilution of bio-MAb (43.3.4.8 or 27.193.3) against either *E. coli* R- and S-LPS. Greater sensitivity of the conjugates against *E. coli* R-LPS was also observed.

2.4 MONOCLONAL ANTIBODY COMPETITION STUDIES

The antigen binding sites of the seven core LPS specific MAbs were compared by adding various combinations of MAbs (one labelled with biotin and one unlabelled) to wells of microtitre plates coated with purified R- or S-LPS. Plates were coated with single LPS types to which unlabelled inhibitor MAbs were known to bind preferentially. Coating with individual, rather than cocktails of LPS gave a clearer indication of MAbs competing for close or overlapping epitopes.

To assess the relatedness of MAb binding sites, a standard curve was established by titrating one MAb against itself, ie, the same antibody was used as both the label and the inhibitor (self-inhibition). The capacity of the unlabelled inhibitor to inhibit the binding of other bio-MAbs was also titrated. All MAb dilutions were made from labelled or unlabelled stocks of approximately 1 mg/ml. Doubling dilutions of

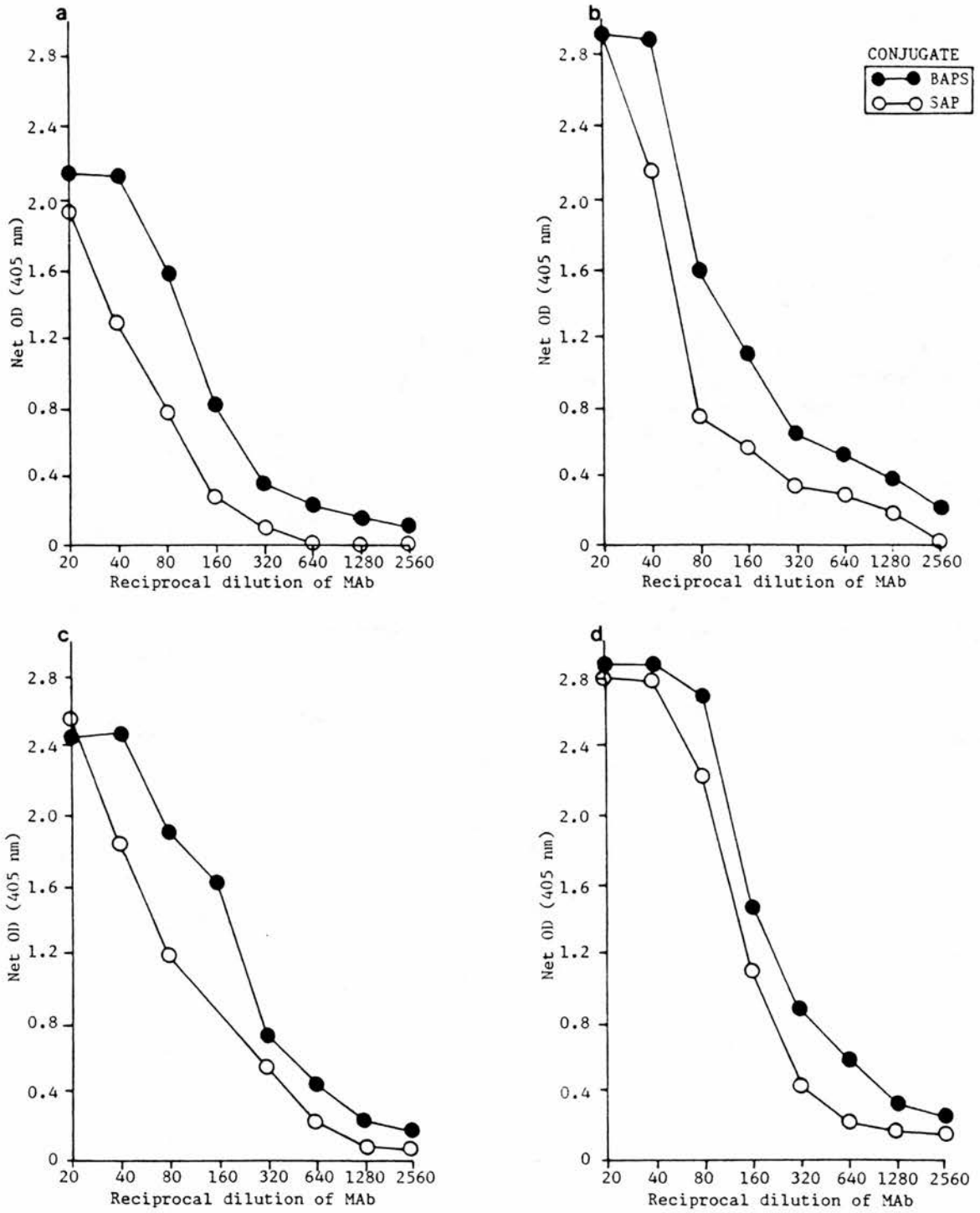


Figure 44. Comparison of the sensitivity of enzyme immunoassays with bio-MAbs 43.3.4.8 (a & b) and 27.193.3 (c & d) against a cocktail of smooth (a & c) and rough (b & d) *E. coli* LPS. The two conjugate systems included: i) alkaline phosphatase labelled biotin streptavidin complex (BAPS) and ii) alkaline phosphatase labelled streptavidin (SAP). The OD values were measured at 405 nm.

bio-MAbs were titrated against doubling dilutions of unlabelled inhibitor: the results for bio-MAbs 43.3.4.8 (diluted 1:160), 43.11.5.1 (diluted 1:80), 43.27.11.2 (diluted 1:40) 27.150.3 (diluted 1:160), 27.193.3 (diluted 1:160), 40.18.7.1 (diluted 1:80) and 30.4.2.8 (diluted 1:160) are shown.

The results were plotted as shown in Figures 45-48 and the concentration of inhibitor required for 50% inhibition of binding of labelled MAbs was compared. A labelled MAb was considered not to compete for the same binding site as the unlabelled inhibitor if the dilution of inhibitor, at the 50% inhibition level was at least one dilution less (ie, inhibitor more concentrated) than required for self-inhibition. In addition, a comparison of self inhibition curves with those representing other combinations of MAbs gave an indication of the relative proximity of MAb binding sites. The degree of inhibition will be dependent on whether or not MAbs share similar binding sites, and the amount of steric hindrance.

MAbs 27.193.3 (Figure 47a) and 30.4.2.8 (Figure 48a) were least capable of inhibiting the binding of all other labelled MAbs. MAbs 43.3.4.8, 43.11.5.1 and 43.27.11.2, previously shown to preferentially bind *E. coli* R1 core, all appeared to bind to different sites. Those MAb combinations showing the greatest inhibition included MAbs 27.150.3, 40.18.7.1 and 43.11.5.1. Although previous results established quite different binding specificities, their respective binding sites are perhaps similarly located on the core antigen.

2.5 CAPTURE ELISA FOR DETECTION OF *E. COLI* LIPOPOLYSACCHARIDE

Capture ELISA systems were developed for the detection of *E. coli* LPS

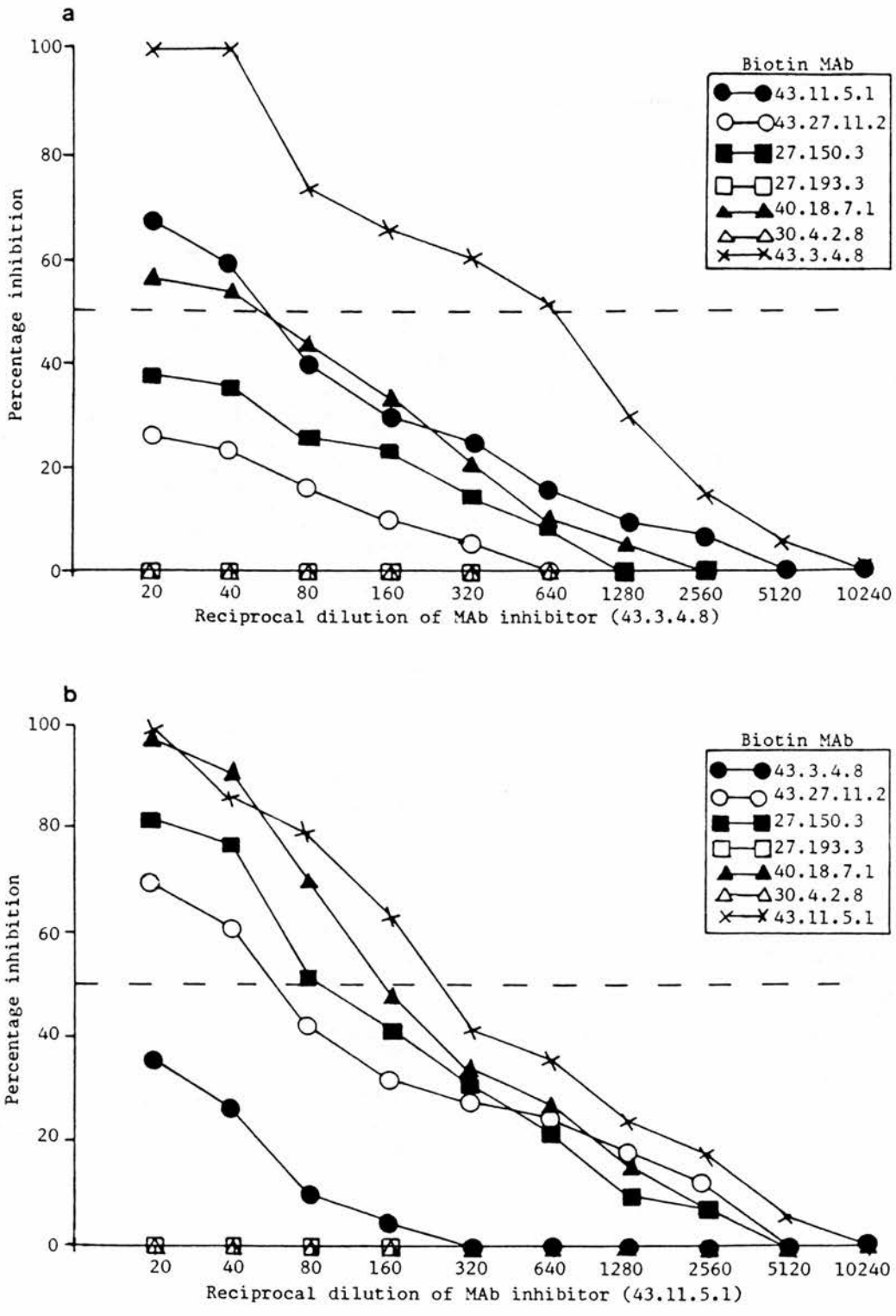


Figure 45. MAb competition assay measured by ELISA (OD 405 nm) with R-LPS from *E. coli* R1 as coating antigen. Doubling dilutions of MAb 43.3.4.8 (a) and MAb 43.11.5.1. (b) inhibitors were run against biotin labelled competing MAbs. The OD values obtained in the presence of inhibitor were calculated as a percentage of OD values obtained without inhibitor, ie, percentage inhibition.

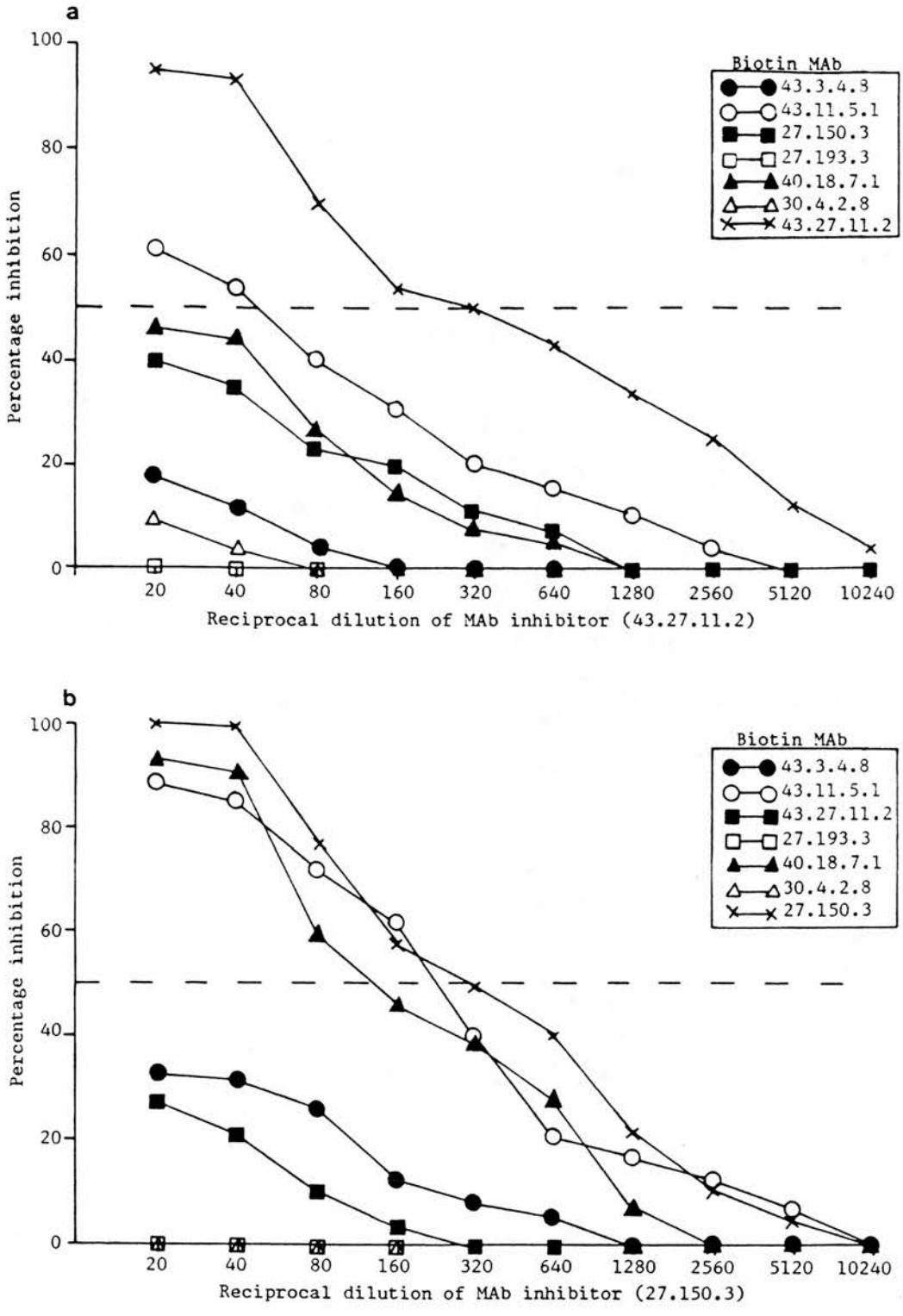


Figure 46. MAb competition assay measured by ELISA (OD 405 nm) with R-LPS from *E. coli* R1 as coating antigen. Doubling dilutions of MAb 43.27.11.2 (a) and MAb 27.150.3 (b) inhibitors were run against biotin labelled competing MAbs. The OD values obtained in the presence of inhibitor were calculated as a percentage of OD values obtained without inhibitor, ie, percentage inhibition.

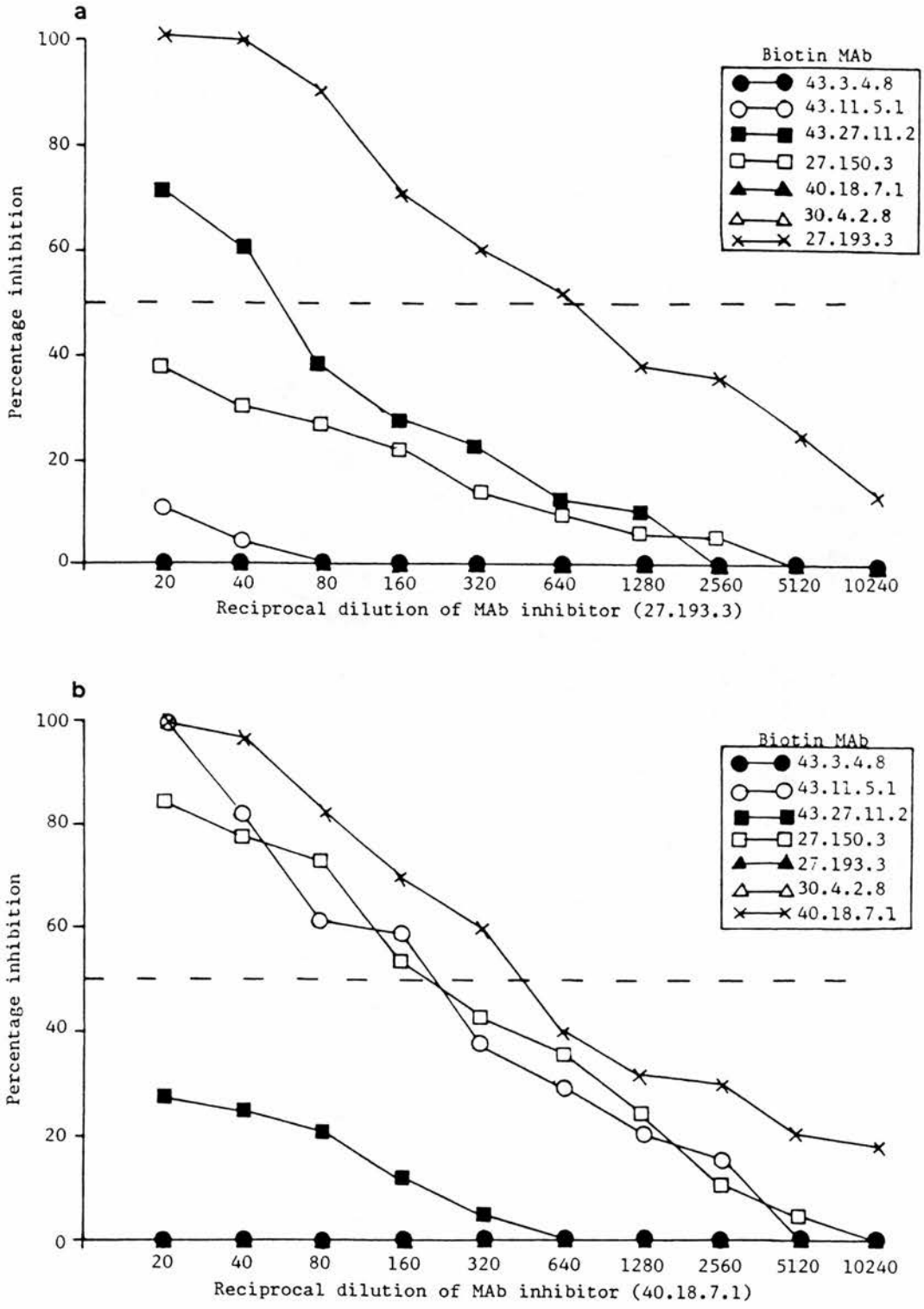


Figure 47. MAb competition assay measured by ELISA (OD 405 nm) with R-LPS from *E. coli* R3 (a) or R2 (b) as coating antigens. Doubling dilutions of MAb 27.193.3 (a) and MAb 40.18.7.1 (b) inhibitors were run against biotin labelled competing MAbs. The OD values obtained in the presence of inhibitor were calculated as a percentage of OD values obtained without inhibitor, ie, percentage inhibition.

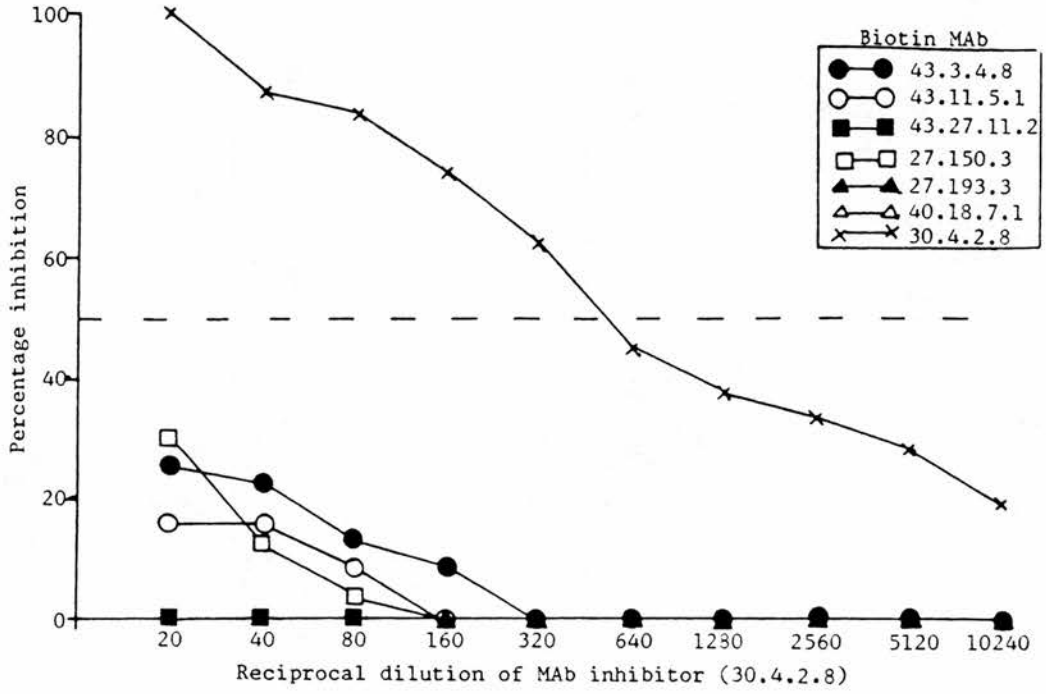


Figure 48. MAb competition assay measured by ELISA (OD 405 nm) with S-LPS from *E. coli* O18 as coating antigen. Doubling dilutions of MAb 30.4.2.8 inhibitor were run against biotin labelled competing MAbs. The OD values obtained in the presence of inhibitor were calculated as a percentage of OD values obtained without inhibitor, ie, percentage inhibition.

in solution. Systems developed included the detection of *E. coli* core types R1-R4, the specific core types of either R1 or R3 and *E. coli* O18 O-antigen. The amounts of coating and conjugate antibodies for each system were determined by chequerboard titrations. Initial investigations compared both the biotin-streptavidin amplification system and the alkaline phosphatase streptavidin system. The sensitivity of each system was similar. Although OD readings were generally higher for the amplification system, background signals were also significantly higher compared to the non-amplified system. The incubation of added antigens for various periods of time (1 h, 2 h, 4 h and overnight) were also investigated; an overnight incubation gave the highest sensitivity for each detection system and was used in all subsequent experiments.

The detection of ten-fold dilutions of *E. coli* LPS and heat-killed cells, incorporating the non-amplification system are shown in Figures 49-52. A sample was considered positive for antigen if it yielded an OD value which was 3 SDs greater than the mean of background controls.

Detection of *E. coli* R1-R4 core types

The system developed incorporated the two most cross-reactive, highest affinity MAbs characterized above: 27.150.3 and 43.11.5.1 which compete for different binding sites. MAb 27.150.3 was used as the coating/capture antibody at 1:200 (approximately 5 *fg* ml^{-1}) and 43.11.5.1 used as the biotinylated antibody (1:1 biotinylation) at 1:100 (approximately 10 *fg* ml^{-1}). The *E. coli* core types of R1-R4 LPS were all detected by this system (Figure 49a). The sensitivity of the assay was between 0.01-10 ng ml^{-1} *E. coli* LPS (0.01 ng ml^{-1} for R1, 0.1 ng ml^{-1} for R2, R4 and O18, and 10 ng ml^{-1} for R3). The assay limits of detection for heat killed whole cell preparations were between 10^4-10^7 cells

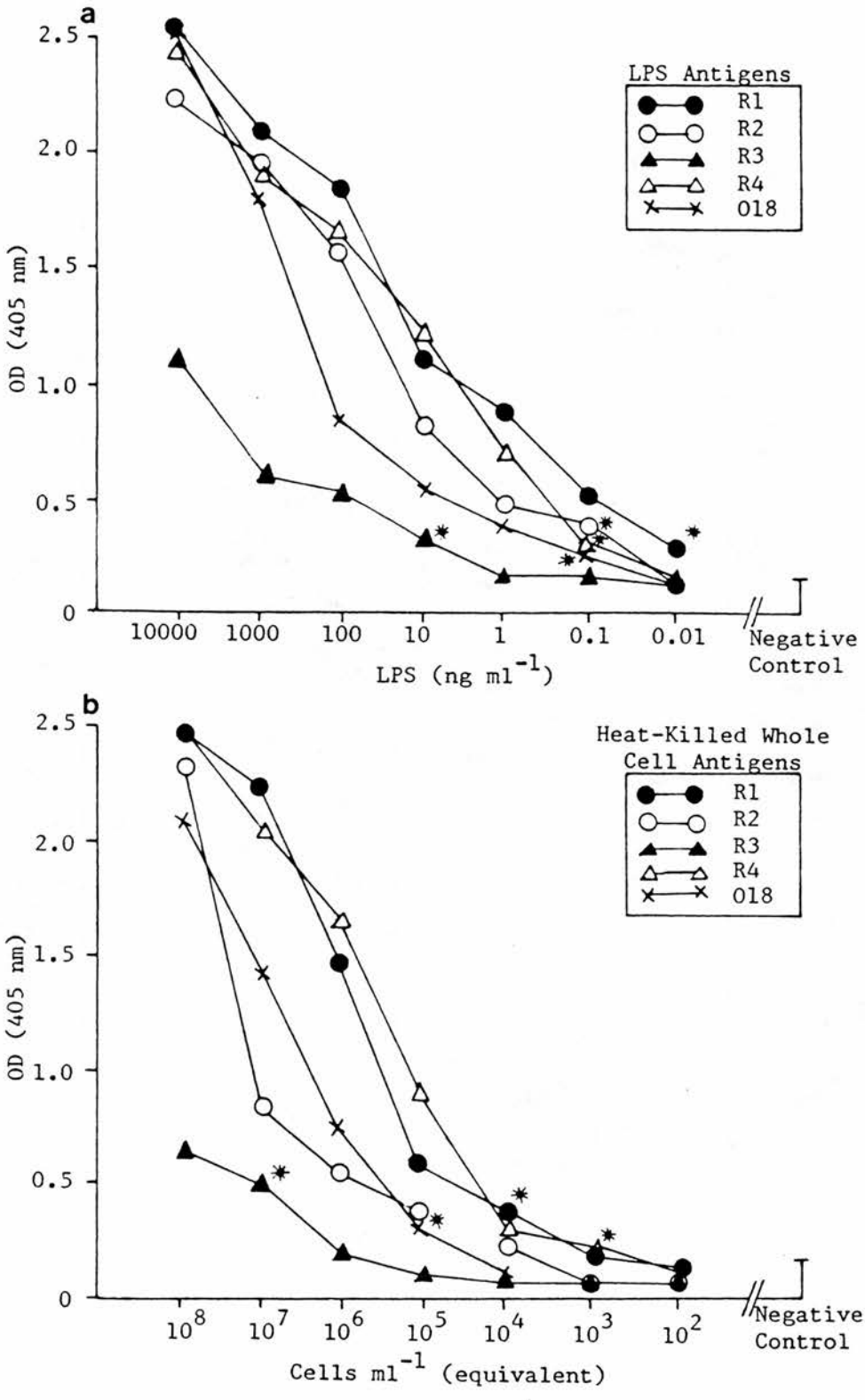


Figure 49. Sensitivity of capture ELISA incorporating an alkaline phosphatase labelled streptavidin system for the detection of a) *E. coli* R1-R4 LPS and b) *E. coli* R1-R4 heat-killed cell preparations. The *E. coli* LPS and heat-killed cell preparations included: *E. coli* core types R1, R2, R3 and R4 (R-LPS), and *E. coli* 018 (S-LPS). Concentrations marked with an asterisk indicate the first value significantly greater than negative control values (>3 SDS of control mean).

equivalent ml^{-1} (Figure 49b).

Other combinations of MAbs were also investigated for the detection of *E. coli* core types R1-R4. These included (i) using the same cross-reactive MAb as both capture and detector antibody, and (ii) the use of a cocktail of core specific MAbs as capture, and a cross-reactive MAb as detector. Although some of these MAb combinations demonstrated comparable, if not greater sensitivity for particular core types compared to the system described above, background signals were high in all cases.

Detection of *E. coli* R1 core type

The specific detection of *E. coli* R1 LPS was achieved using MAb 43.3.4.8 which reacts almost exclusively with the R1 core, and the cross-reactive MAb 43.11.5.1. Both MAbs were shown not to compete for similar binding sites. MAb 43.3.4.8 was used as the coating/capture antibody at 1:100 (approximately 10 $\mu g\ ml^{-1}$) and 43.11.5.1 was used as the biotinylated antibody (1:1 biotinylation) at 1:100 (approximately 10 $\mu g\ ml^{-1}$). The sensitivity of the assay was 0.01 ng ml^{-1} for the detection of *E. coli* R1 (R-LPS) and 0.1 ng ml^{-1} for the R1 containing core of *E. coli* 018 (S-LPS) (Figure 50a). Other *E. coli* core types were not detected in the assay. The sensitivity of the assay for the detection of *E. coli* R1 (R-LPS) and 018 (S-LPS) heat-killed cell preparations was 10^4 and 10^5 cells equivalent ml^{-1} respectively (Figure 50b).

Detection of *E. coli* R3 core type

MAb 27.193.3 was used as both the capture/coating antibody and biotinylated antibody (1:1 biotinylation) for the specific detection of

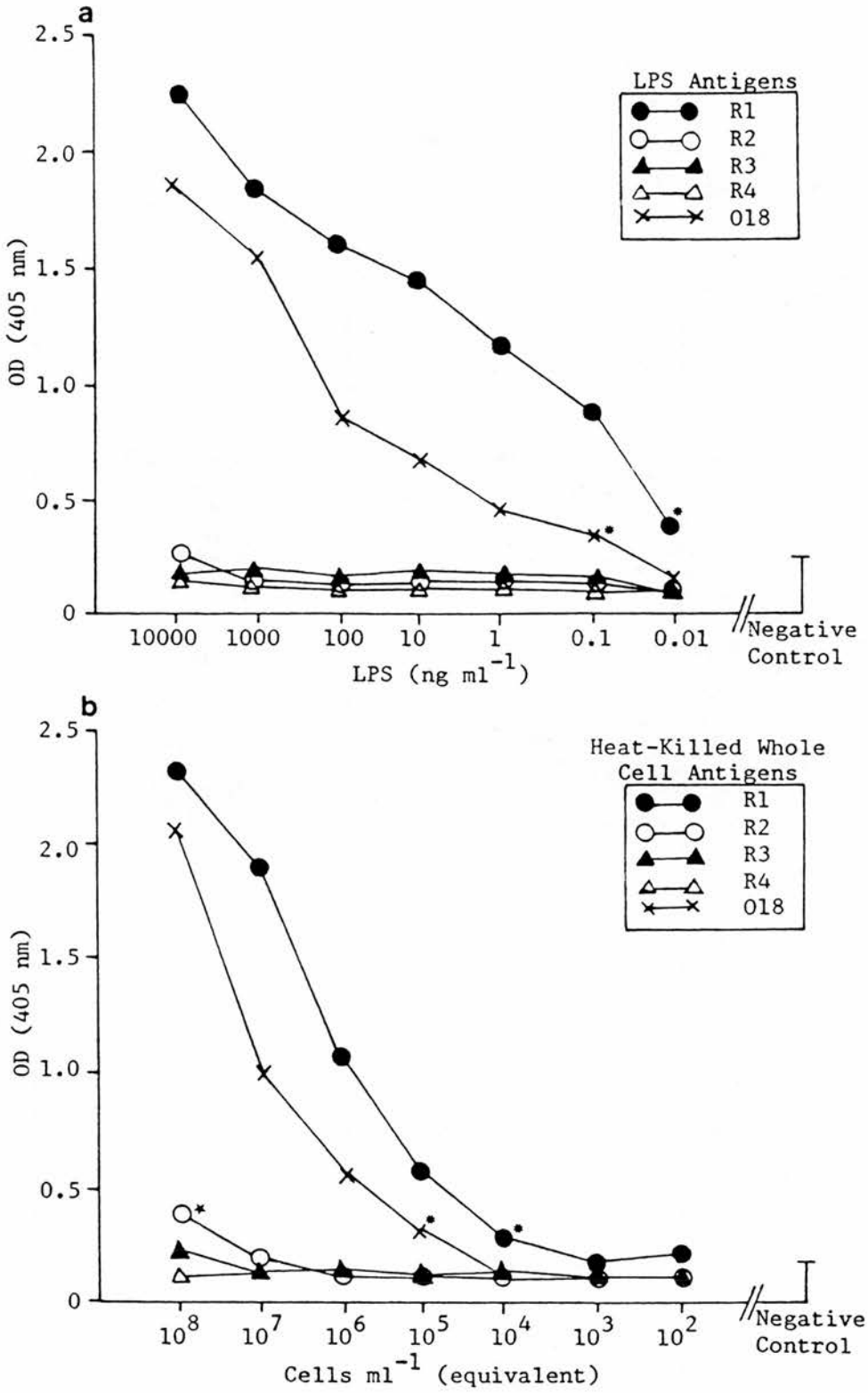


Figure 50. Sensitivity of capture ELISA incorporating an alkaline phosphatase labelled streptavidin system for the detection of a) *E. coli* R1 LPS and b) *E. coli* R1 heat-killed cell preparations. The *E. coli* LPS and heat-killed cell preparations included: *E. coli* core types R1, R2, R3 and R4 (R-LPS), and *E. coli* 018 (S-LPS). Concentrations marked with an asterisk indicate the first value significantly greater than negative control values (>3 SDS of control mean).

E. coli R3 LPS. Both capture and conjugate antibodies were used at 1:200 (approximately 5 $\mu g\ ml^{-1}$). The sensitivity of the assay was 0.01 ng ml^{-1} for the detection of *E. coli* R3 (R-LPS) and 0.1 ng ml^{-1} for the R3 containing core of *E. coli* 015 (S-LPS) (Figure 51a). The assay also detected a minimum of 10^4 and 10^6 cells equivalent ml^{-1} of the heat-killed cell preparations of *E. coli* R3 (R-LPS) and 015 (S-LPS) respectively (Figure 51b). The assay was unable to detect all other *E. coli* core types apart from R1, which was detected at only the highest concentration of the LPS and heat-killed cell preparations.

Detection of *E. coli* 018 O-antigen

The specific detection of *E. coli* 018 O-antigen used MAb 30.4.2.8 as both the capture/coating antibody and biotinylated antibody (1:1 biotinylation). Both capture and conjugate antibodies were used at 1:100 (approximately 10 $\mu g\ ml^{-1}$). The sensitivity of the assay was 0.01 ng ml^{-1} for the detection of LPS from *E. coli* 018 (S-LPS) (Figure 52a) and 10^3 cells equivalent ml^{-1} for the detection of *E. coli* 018 heat-killed cell preparation (Figure 52b). Positive values were also obtained with R1 and 015 antigen preparations at the highest concentrations only.

Detection of LPS in spiked serum

The capture ELISA systems were also applied for the detection of *E. coli* LPS in spiked human serum samples, known to contain low levels of core-glycolipid antibodies. Preliminary studies established high backgrounds and low sensitivity with normal untreated serum. Various methods were investigated in an attempt to improve assay sensitivity for serum spiked with ten-fold dilutions of *E. coli* LPS:

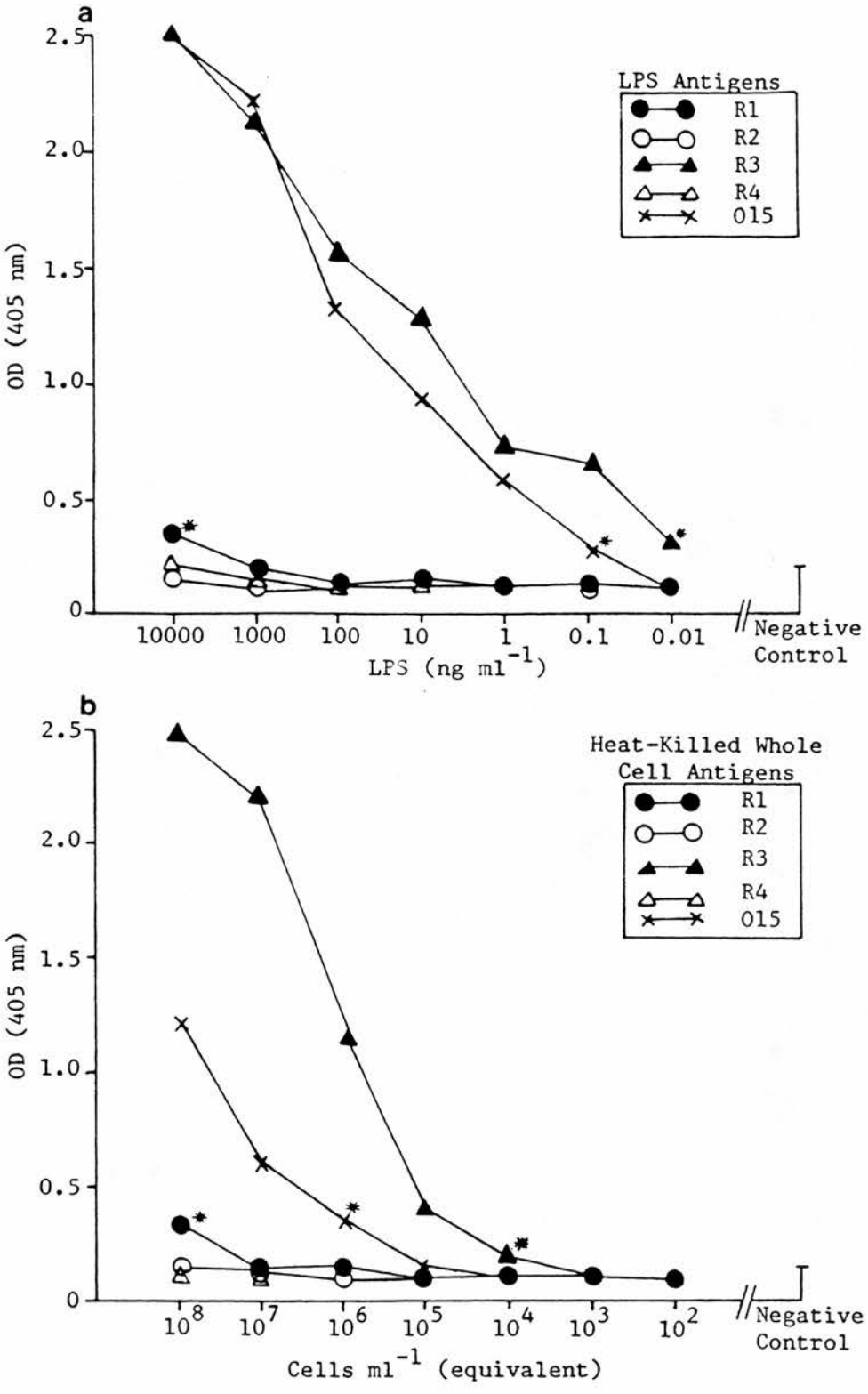


Figure 51. Sensitivity of capture ELISA incorporating an alkaline phosphatase labelled streptavidin system for the detection of a) *E. coli* R3 LPS and (b) *E. coli* R3 heat-killed cell preparations. The *E. coli* LPS and heat-killed cell preparations included: *E. coli* core types R1, R2, R3 and R4 (R-LPS), and *E. coli* 015 (S-LPS). Concentrations marked with an asterisk indicate the first value significantly greater than negative control values (>3 SDS of control mean).

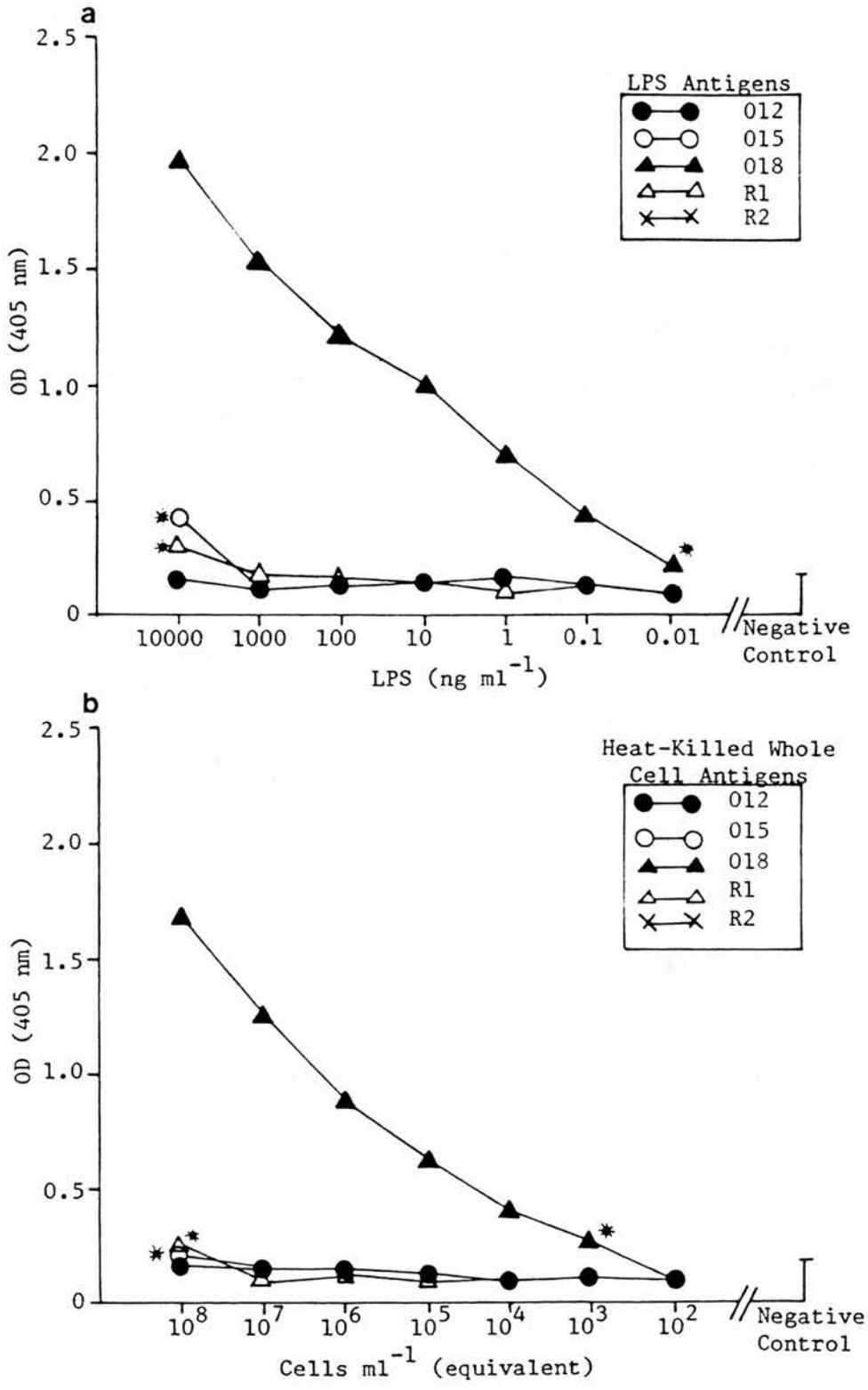


Figure 52. Sensitivity of capture ELISA incorporating an alkaline phosphatase labelled streptavidin system for the detection of a) *E. coli* 018 LPS and b) *E. coli* 018 heat-killed cell preparations. The *E. coli* LPS and heat-killed cell preparations included: *E. coli* core types R1 and R2 (R-LPS), and *E. coli* 012, 015 and 018 (S-LPS). Concentrations marked with an asterisk indicate the first value significantly greater than negative control values (>3 SDS of control mean).

(i) Spiked samples were diluted 1:4 with pyrogen-free 0.15 M NaCl and incubated for 30 min in a 60°C water bath (Roth *et al*, 1990).

(ii) Spiked samples were diluted 1:5 in pyrogen-free water and heated for either 30 min at 56°C or 3 min at 100°C.

(iii) Spiked samples were treated by an alkaline denaturation method described by (Limet *et al*, 1988). Samples were diluted 1:4 in 0.1 M NaOH and heated for 30 min at 70°C. The mixture was then neutralized (pH 8.5) by one volume of 0.8 M boric acid.

(iv) Spiked samples were diluted 1:2 in pyrogen-free water and the LPS extracted using phenol. Equal volumes of prewarmed (67°C) sample and phenol solution (90 w/v) were mixed and stirred at 67°C for 15 min. The mixture was cooled in ice until phase separation occurred before contrifuging at 5,000 g for 15 min to complete the process. The upper acqueous phase containing the LPS was transferred to dialysis tubing and dialysed against running water overnight to remove phenol.

All treated samples were diluted 1:2 in ELISA diluent and added at 200 μl per MAb coated well.

The effects of the above serum treatments on the sensitivity of the capture ELISA for the specific detection of *E. coli* R1 (capture/coating MAb 43.3.4.8, biotinylated MAb 43.11.5.1) are shown in Table 7. The sensitivity of the assay for the detection of LPS in spiked serum was at its optimum following dilution of serum four-fold with 0.15 M NaCl and heating for 30 min at 60°C (mean 4 ± 5.2 ng ml^{-1}), and after the phenol extraction method (mean 36.7 ± 55 ng ml^{-1}). The sensitivity of

Table 7. The sensitivity of a capture ELISA assay for the detection of *E. coli* R1 LPS in spiked serum after various treatments.

Detection of *E. coli* R1 in spiked serum

Treatment	Mean sensitivity (ng ml^{-1}) (±SD)*	
Control (+ve): LPS diluted in ELISA diluent	0.04	(0.052)
Control (-ve): LPS diluted in untreated serum	400	(519.6)
Spiked serum diluted 1:4 with 0.15 M NaCl, heated 30 min at 60°C	4	(5.2)
Spiked serum diluted 1:5 with H_2O and heated for:		
i) 30 min at 56°C	70	(52)
ii) 3 min at 100°C	700	(520)
Spiked serum diluted 1:4 with 0.1 N NaOH, heated 30 min at 70°C and neutralized (pH 8.5) with 0.8 M boric acid	70	(52)
Phenol extraction: spiked serum diluted 1:2 with H_2O, mixed with equal volume of phenol (90% w/v). Aqueous phase removed and dialysed.	36.7	(55)

* Each value represents the mean (±SD) from three separate experiments.

the assay for LPS diluted in ELISA diluent was 0.04 ± 0.052 ng ml^{-1}. The use of capture ELISAs employing other MAb combinations for the detection of specific LPSs in serum, produced similar results (data not shown).

CHAPTER 3

MONOCLONAL ANTIBODIES AS PROBES FOR DETECTING LIPOPOLYSACCHARIDE EXPRESSION ON *ESCHERICHIA COLI* FROM DIFFERENT GROWTH CONDITIONS

The purpose of this work was to use MAbs as probes to investigate the expression of LPS on *E. coli* strains, grown under a variety of nutrient conditions in batch culture which mimicked some of the *in vivo* environmental conditions of an infected host. A nitrogen deficient/ high carbon medium was also used to promote capsule production (Sutherland & Wilkinson, 1965). The presence and absence of capsule on selected *E. coli* strains was confirmed by phage typing and whole cell electron microscopy (Figure 53). Growth under nitrogen deficient/high carbon conditions appeared to improve the expression of the *E. coli* K1 capsule (approximately 250 nm thick compared to 125 nm under nutrient broth conditions).

The expression of LPS on bacteria grown *in vivo* was also investigated.

3.1 SILVER STAINING AND IMMUNOBLOT ANALYSIS OF LIPOPOLYSACCHARIDES FROM BACTERIA GROWN *IN VITRO*

Figures 54-65 include silver stained LPS PAGE profiles and their corresponding immunoblots from four *E. coli* strains grown under different growth conditions. The three smooth strains showed the characteristic ladder pattern, each step up representing LPS substituted with a progressively increasing number of O-polysaccharide repeating oligosaccharide units. These were missing from the rough mutant. The effect of each growth condition on the expression of LPS was compared with nutrient broth.

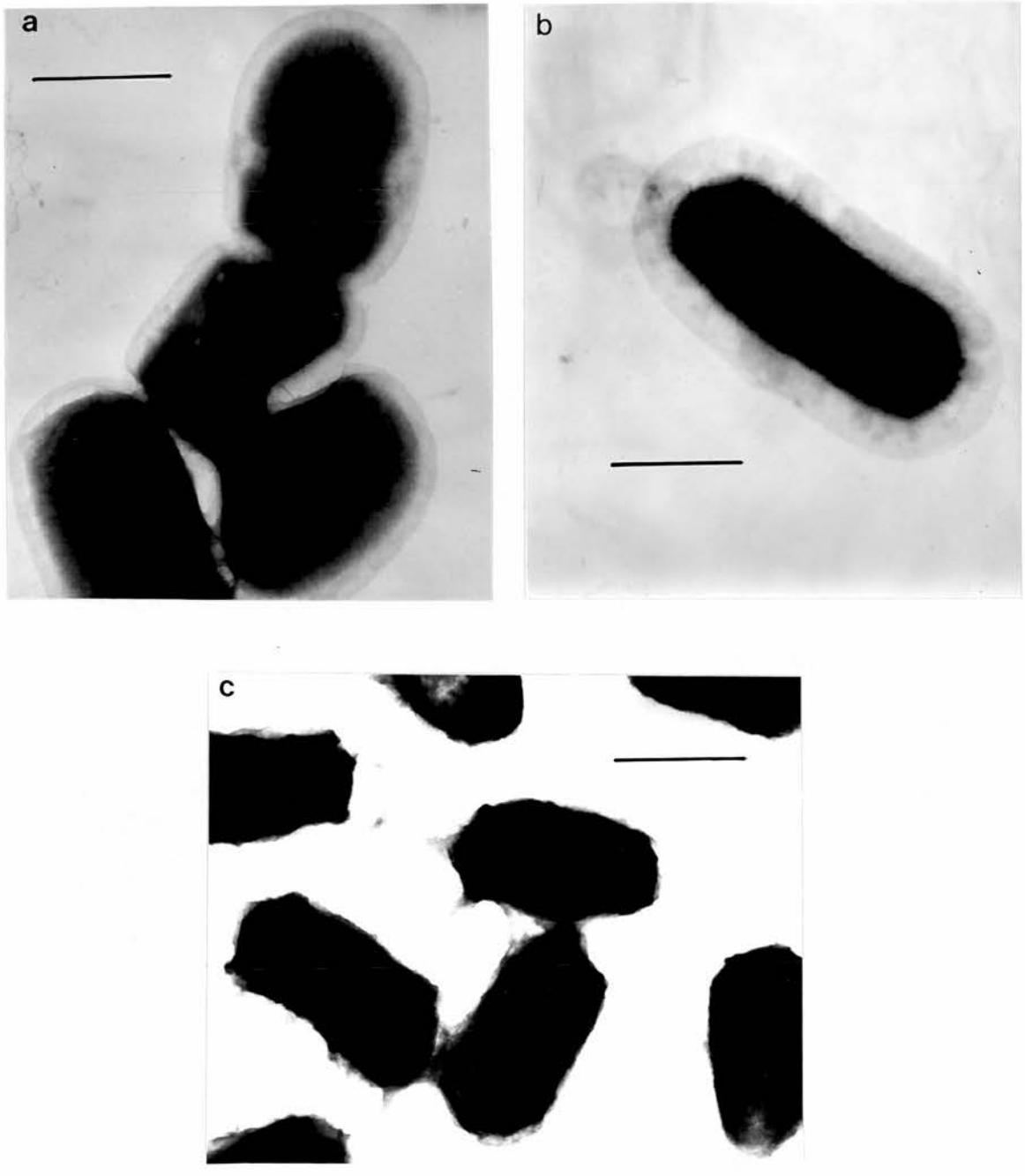


Figure 53. Electron micrographs of negatively stained whole cells of *E. coli* 018:K1 grown in nutrient broth (a) and nitrogen deficient/high carbon medium (b). c) represents the non-capsulate mutant 018:K1 grown in nutrient broth. Bars, 1 μm.

Silver stained LPS profiles of proteinase K digested whole cells grown in nutrient broth, iron depleted and nitrogen deficient/high carbon media, are shown in Figure 54. LPS from iron depleted cells show only minor changes against the LPS from nutrient broth grown cells, whilst cells grown in a nitrogen deficient/high carbon medium showed an increased expression of both mid-range and high molecular mass bands. Immunoblotting of transblotted gels against O18, O-antigen specific MAb 184.2.5.5 (Figure 55a) demonstrates greater expression of O-polysaccharide as well as an overall increase in its chain length for cells grown under nitrogen deficiency. Probing with 27.150.3, an anti-core MAb, reactive against both substituted and unsubstituted core material showed no significant differences between growth conditions (Figure 55b). The same LPS samples were separated by either a non-gradient 14% acrylamide gel or a gradient 5-20% acrylamide gel and immunoblotted against anti-core MAb 43.27.11.2. Figure 56a demonstrates the reactivity of the MAb against unsubstituted core material and the high molecular mass substituted core material leaking from the rough mutant of *E. coli* O18. Marginally stronger reactivity was observed against *E. coli* strains prepared under iron depletion. The gradient gel (Figure 56b) improved the resolution of the low molecular mass LPS bands. MAb 43.27.11.2 was shown to be reactive in most cases against at least three low molecular mass bands of both R- and S-LPS strains. Reactivity against *E. coli* strains grown under iron depletion was significantly greater than nutrient broth or nitrogen deficient/high carbon conditions.

E. coli strains were grown in iron depleted media containing different concentrations of the iron-chelator 2,2' dipyridyl (25, 50, 100, 150 and 200 μmol. L^{-1}). The effect of increasing the iron-chelator

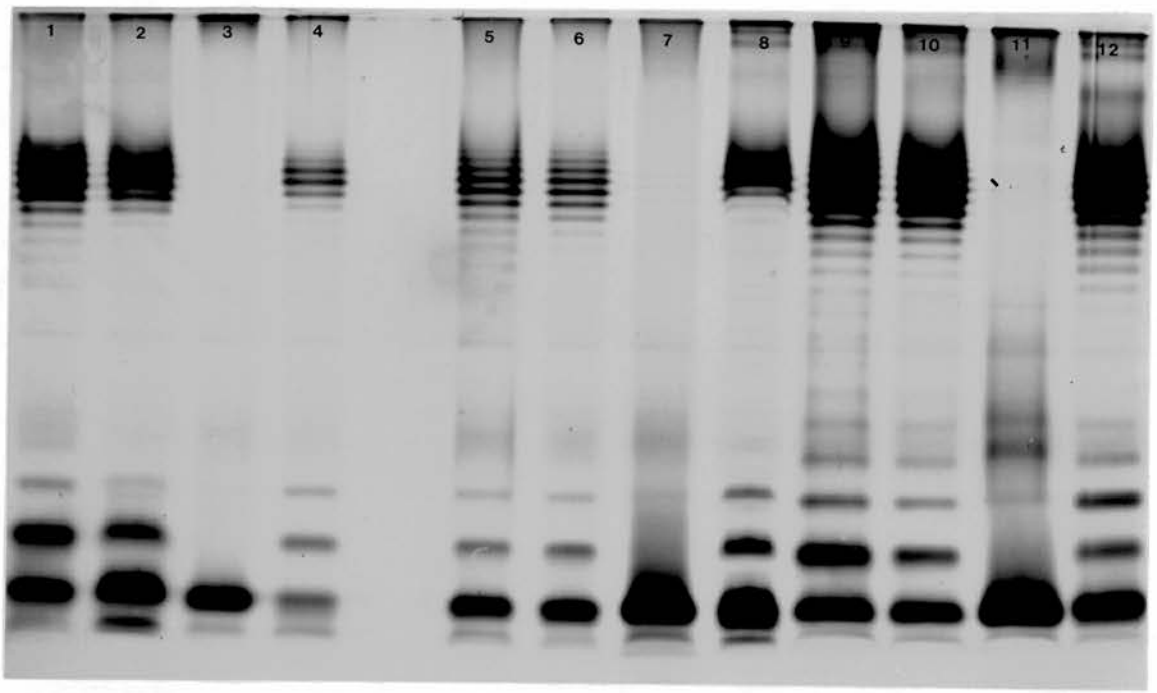
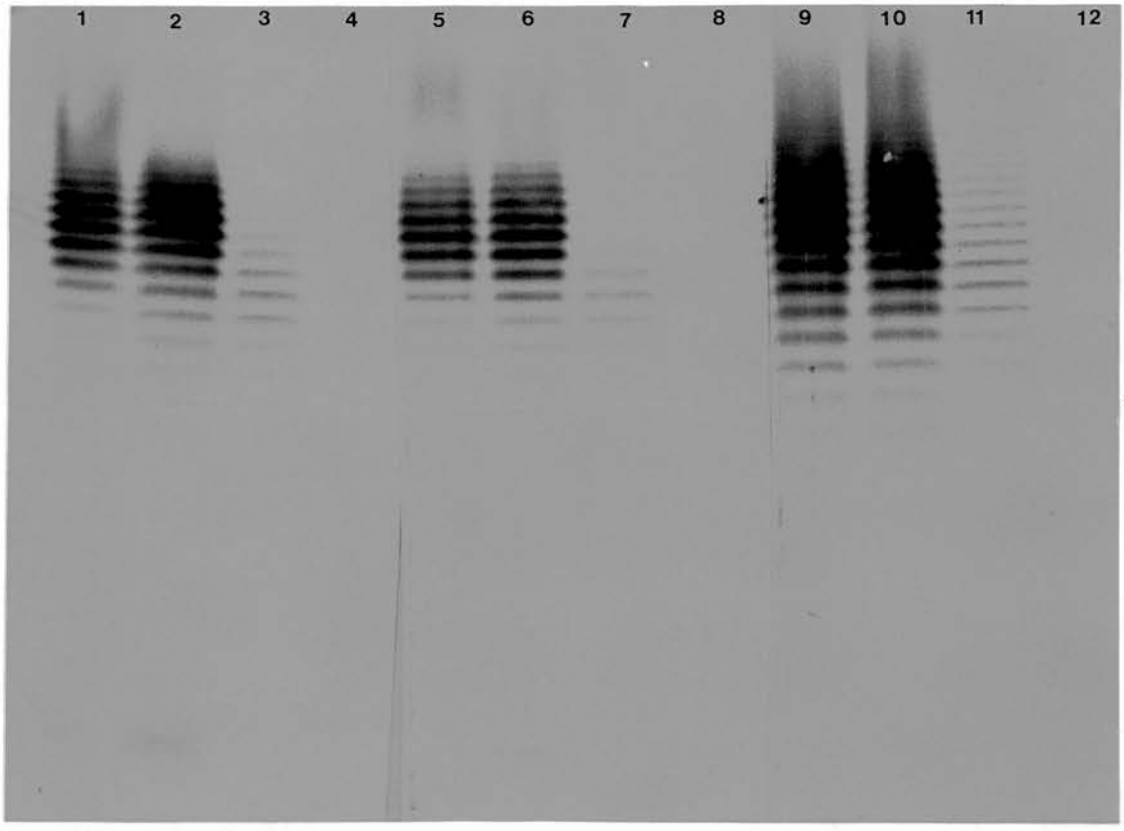


Figure 54. Silver stained LPS profiles of proteinase K whole cell digests of *E. coli* strains separated by PAGE 14% w/v acrylamide). Tracks 1-4, 5-8 and 9-12 represent 4 *E. coli* strains (O18:K1, O18:K1 , O18:Krf and O6:K5) grown to early stationary phase in nutrient broth, an iron depleted medium (containing 150 μmol. L^{-1} 2,2' dipyridyl) and a nitrogen deficient/high carbon medium respectively.

a



b

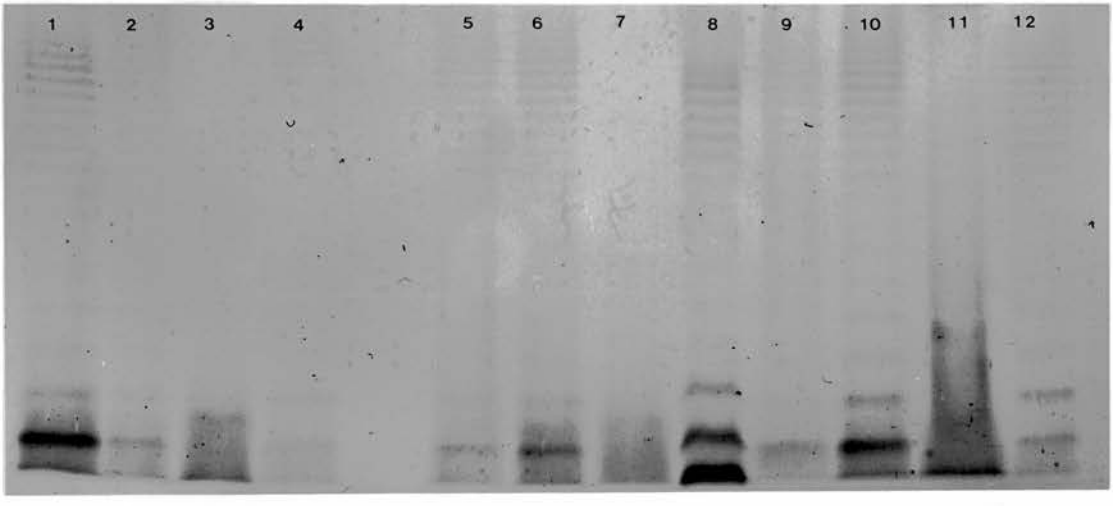


Figure 55. Immunoblots of proteinase K whole cell digests of *E. coli* strains separated by PAGE (14% w/v acrylamide) followed by transfer to NIC paper and probed with a) an O18 O-antigen specific MAb, 184.2.5.5 and b) a core-specific MAb, 27.150.3 Tracks 1-4, 5-8 and 9-12 represent 4 *E. coli* strains (O18:K1, O18:K1$^-$, O18:Krf and O6:K5) grown to early stationary phase in nutrient broth, an iron depleted medium (containing 150 μmol. L^{-1} 2,2' dipyridyl) and a nitrogen deficient/high carbon medium respectively.

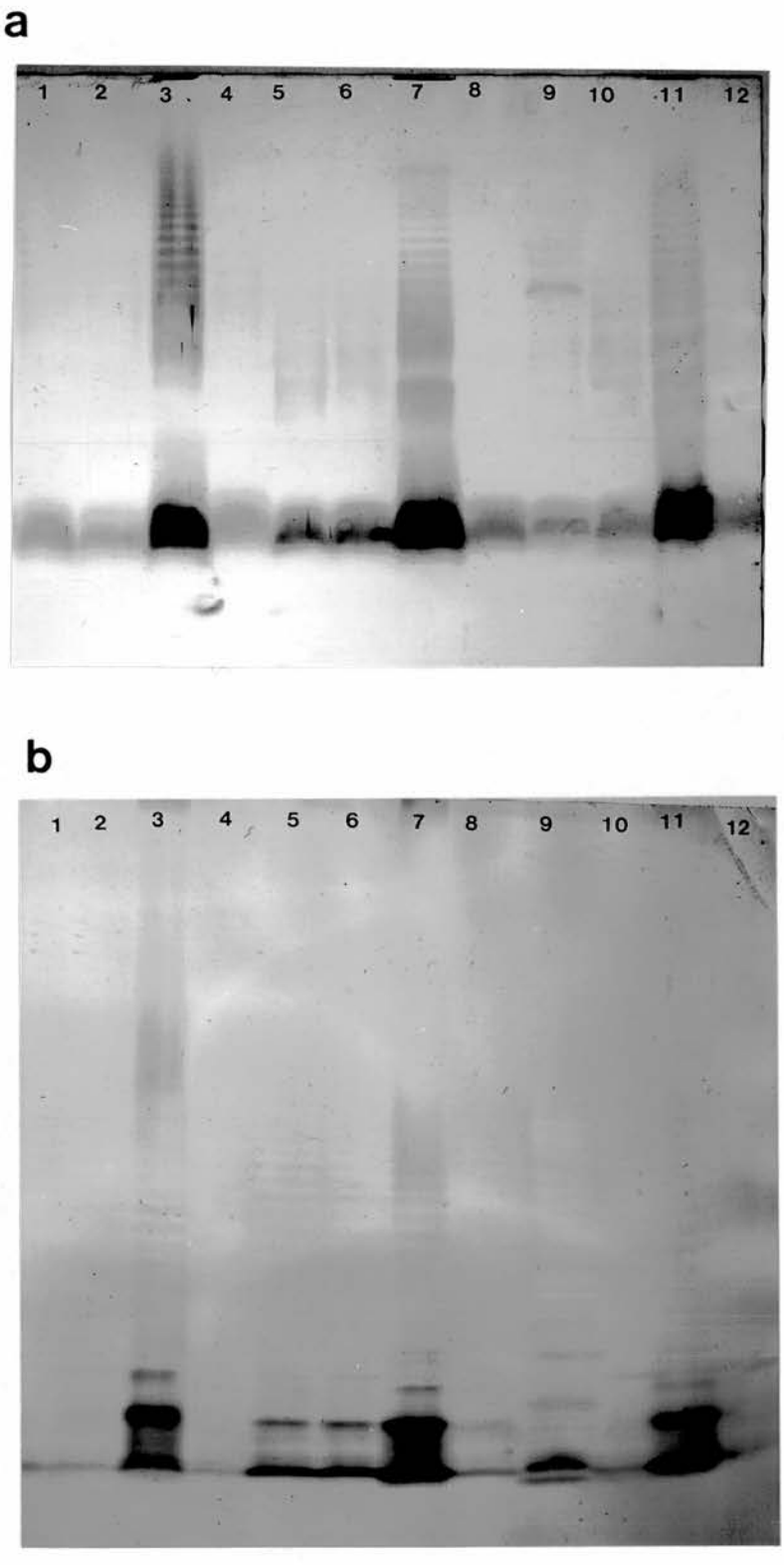


Figure 56. Immunoblots of proteinase K whole cell digests of *E. coli* strains separated by PAGE (a) 14% w/v acrylamide and b) 5-20% w/v acrylamide gradient) followed by transfer to NIC paper and probed with a core-specific MAb, 43.27.11.2. Tracks 1-4, 5-8 and 9-12 represent 4 *E. coli* strains (O18:K1, O18:K1^{-}, O18:Krf and O6:K5) grown to early stationary phase in nutrient broth, an iron depleted medium (containing 150 μmol. L^{-1} 2,2' dipyridyl) and a nitrogen deficient/high carbon medium respectively.

concentration on the growth of *E. coli* strain O18:K1 is shown in Figure 57. The lag phase increased, growth rate decreased and final cell concentration as measured by absorbance decreased with an increase in iron-chelator concentration. Figure 58 represents silver stained PAGE profiles of LPS and their corresponding immunoblots from four *E. coli* strains grown to early stationary phase under conditions described above. An increase in iron-chelator concentration was associated with a decreased expression of O-antigen bearing molecular mass bands of S-LPS, as illustrated by silver stained profiles (Figure 58a) and the immunoblot against O18, O-antigen specific MAb 184.2.5.5 (Figure 58c). Probing with a core-specific MAb, 43.27.11.2 showed increased reactivity against S-LPS strains grown in the presence of 150 and 200 μmol L^{-1} 2,2' dipyridyl compared to the lower concentrations of 25, 50 and 100 μmol L^{-1} (Figure 58b).

Modifications of Malka minimal medium containing 1% and zero volume of the magnesium salt used for the standard medium (magnesium depleted [0.17 mmol. L^{-1}] and deficient growth media respectively) were used for the growth of *E. coli* strains. Silver stained LPS profiles of four magnesium deficient and depleted *E. coli* strains showed a significant increase in the expression of unsubstituted core-glycolipid material and other low molecular mass bands compared to growth in nutrient broth (Figure 59). Immunoblotting with core-specific MAb 27.150.3 further highlighted this pronounced increase in core LPS (Figure 60a), whilst probing with MAb 184.2.5.5 showed similar expression of O-antigen for both nutrient broth and magnesium depleted conditions (Figure 60b).

The silver stained LPS profiles of *E. coli* strains grown under magnesium/iron depleted conditions showed an increased expression of

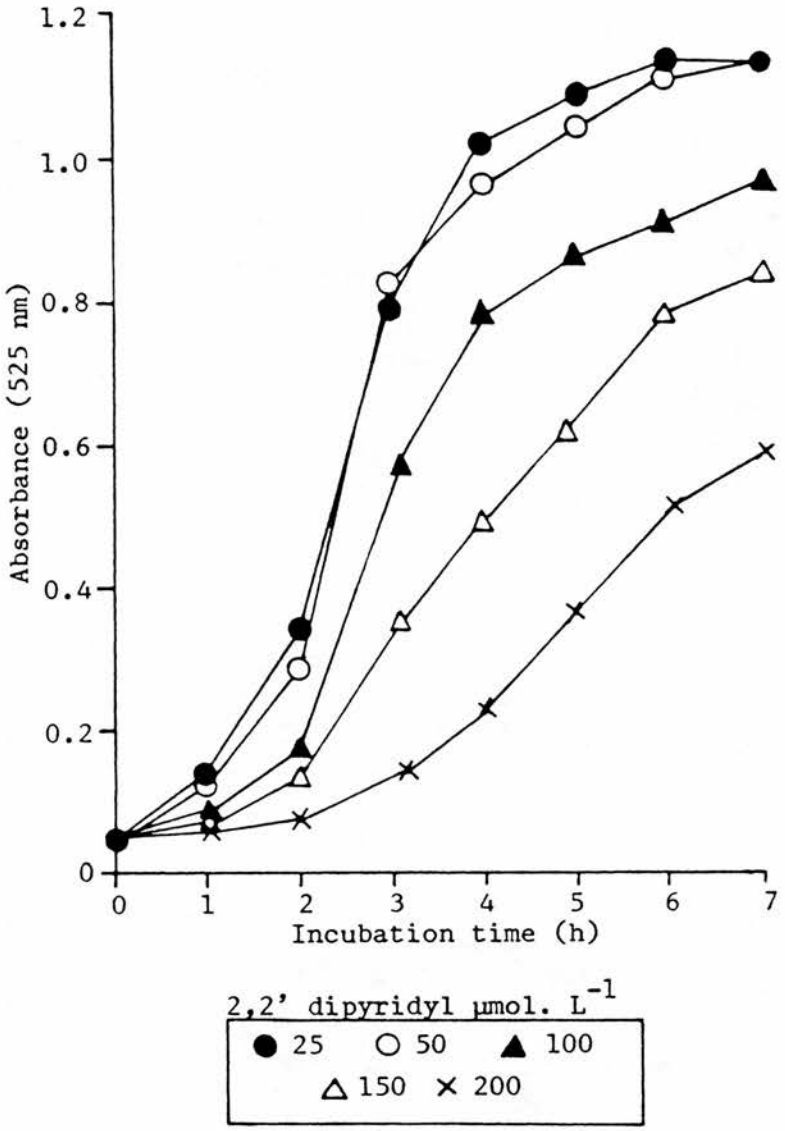


Figure 57. Growth of *E. coli* 018:K1 in iron depleted media containing 25, 50, 100, 150 and 200 µmol. L^{-1} 2,2' dipyridyl as determined by measurement of absorbance of bacterial suspension at 525 nm.

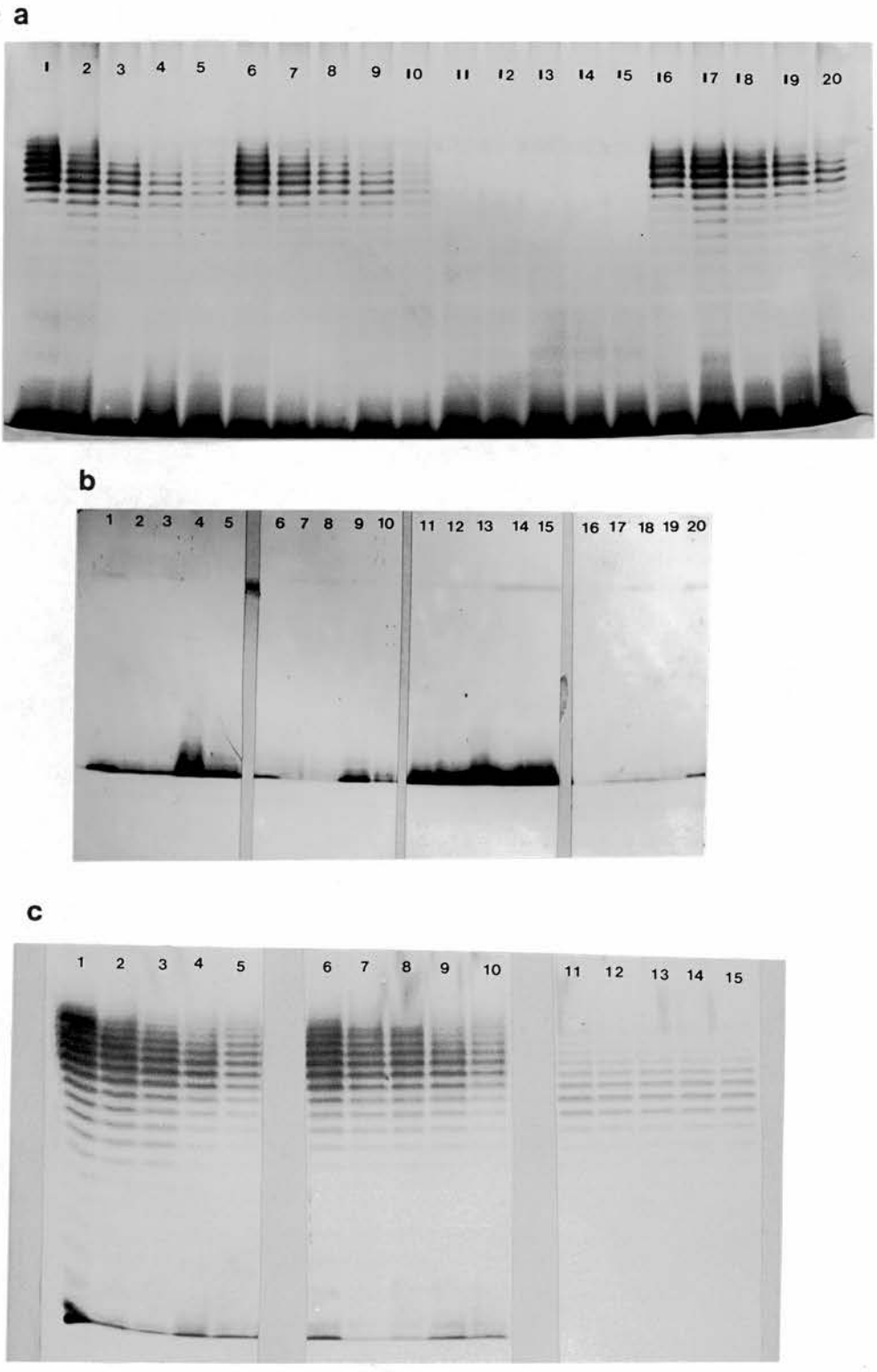


Figure 58. a) Silver stained LPS profiles of proteinase K whole cell digests of *E. coli* strains separated by PAGE (14% w/v acrylamide). (b & c) Immunoblots following transfer of LPS to NIC paper and probing with b) a core-specific MAb 43.27.11.2 and c) an O18 O-antigen specif MAb, 184.2.5.5. Tracks, 1-5, 6-10, 11-15 and 16-20 represent *E. coli* strains O18:K1, O18:K1 , O18:Krf and O6:K5 respectively. Each strain was grown to early stationary phase in iron depleted media containing 25, 50, 100, 150 and 200 μmol. L^{-1} 2,2' dipyridyl.

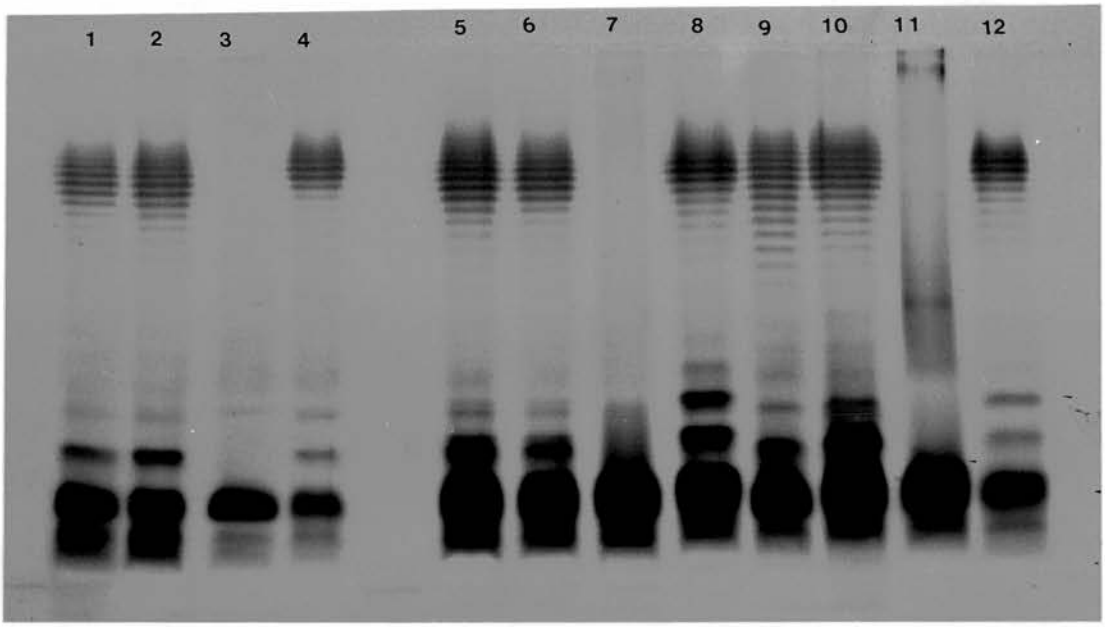
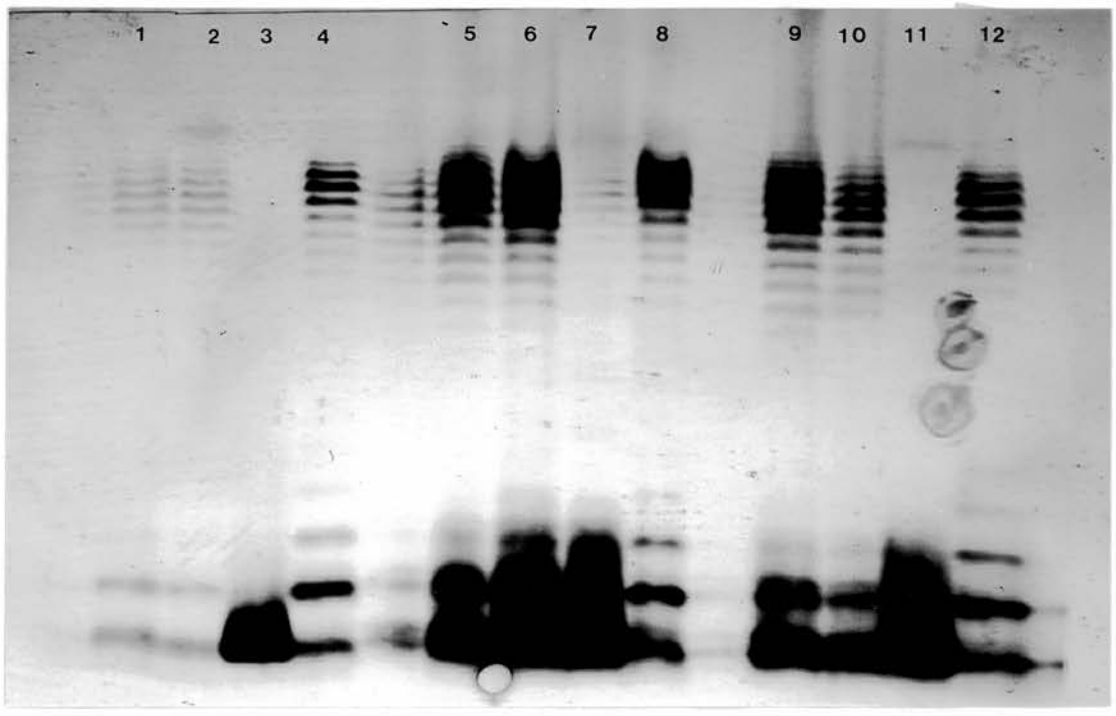


Figure 59. Silver stained LPS profiles of proteinase K whole cell digests of *E. coli* strains separated by PAGE (14% w/v acrylamide). Tracks 1-4, 5-8 and 9-12 represent 4 *E. coli* strains (O18:K1, O18:K1 , O18:Krf and O6:K5) grown to early stationary phase in nutrient broth and a magnesium deficient or depleted medium (containing either zero or 1% volume of the magnesium salt used for the Malka minimal medium) respectively.

a



b

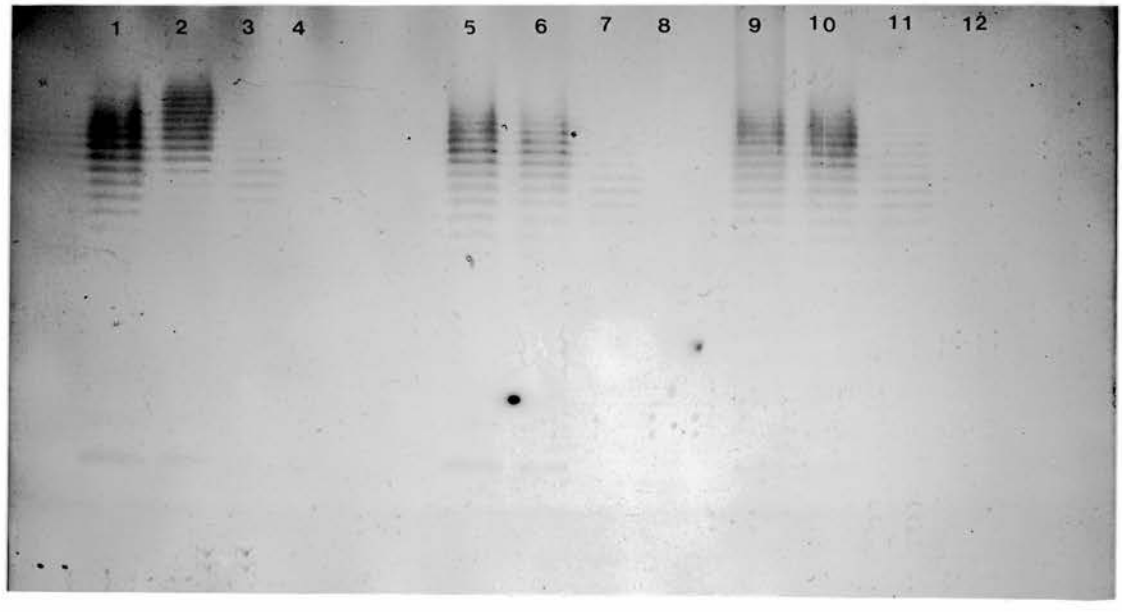


Figure 60. Immunoblots of proteinase K whole cell digests of *E. coli* strains separated by PAGE (14% w/v acrylamide) followed by transfer to NIC paper and probed with a) a core-specific MAb, 27.150.3 and b) an O18 O-antigen specific MAb 184.2.5.5. Tracks 1-4, 5-8 and 9-12 represent 4 *E. coli* strains (O18:K1, O18:K1 , O18:Krf and O6:K5) grown to early stationary phase in nutrient broth and a magnesium deficient or depleted medium (containing either zero or 1% volume of the magnesium salt used for the Malka minimal medium) respectively.

low molecular mass unsubstituted core material (Figure 61a). The corresponding immunoblot against anti-core MAb, 43.27.11.2 further demonstrated the heightened expression of core LPS (Figure 61b).

Growth in heat-inactivated sheep serum resulted in greater expression of both the fast migrating core region, and the high molecular mass, O-antigen bearing molecular species of the smooth strains, compared to growth in nutrient broth (Figure 62a). Immunoblot analysis with MAb 184.2.5.5 again illustrates an increased expression of O-antigen (Figure 62b). Immunoblot analysis with anti-core MAbs 27.150.3 and 43.27.11.2 demonstrates increased expression of predominantly rough form LPS grown in serum (Figure 63a & b).

The growth curves of *E. coli* strains O18:K1 and O18:K1$^-$ cultured in nutrient broth, heat-inactivated sheep serum and untreated sheep serum are presented in Figure 64. Both organisms divided more rapidly in heat-inactivated sheep serum than untreated sheep serum. The growth curves for organisms grown in heat-inactivated sheep serum were similar to those obtained for growth in nutrient broth.

Silver stained LPS PAGE profiles of nutrient broth, heat-inactivated sheep serum and untreated sheep serum culture samples (removed at hourly intervals), are shown in Figure 65 for both *E. coli* O18:K1 a) and O18:K1$^-$ b). Greater expression of unsubstituted core and O-antigen bearing bands with increasing incubation time was observed for both organisms grown under the different nutrient conditions. The expression of core and O-antigen after each period of incubation was greater for organisms grown in heat-inactivated sheep serum and untreated sheep serum than the equivalent samples grown in nutrient broth.

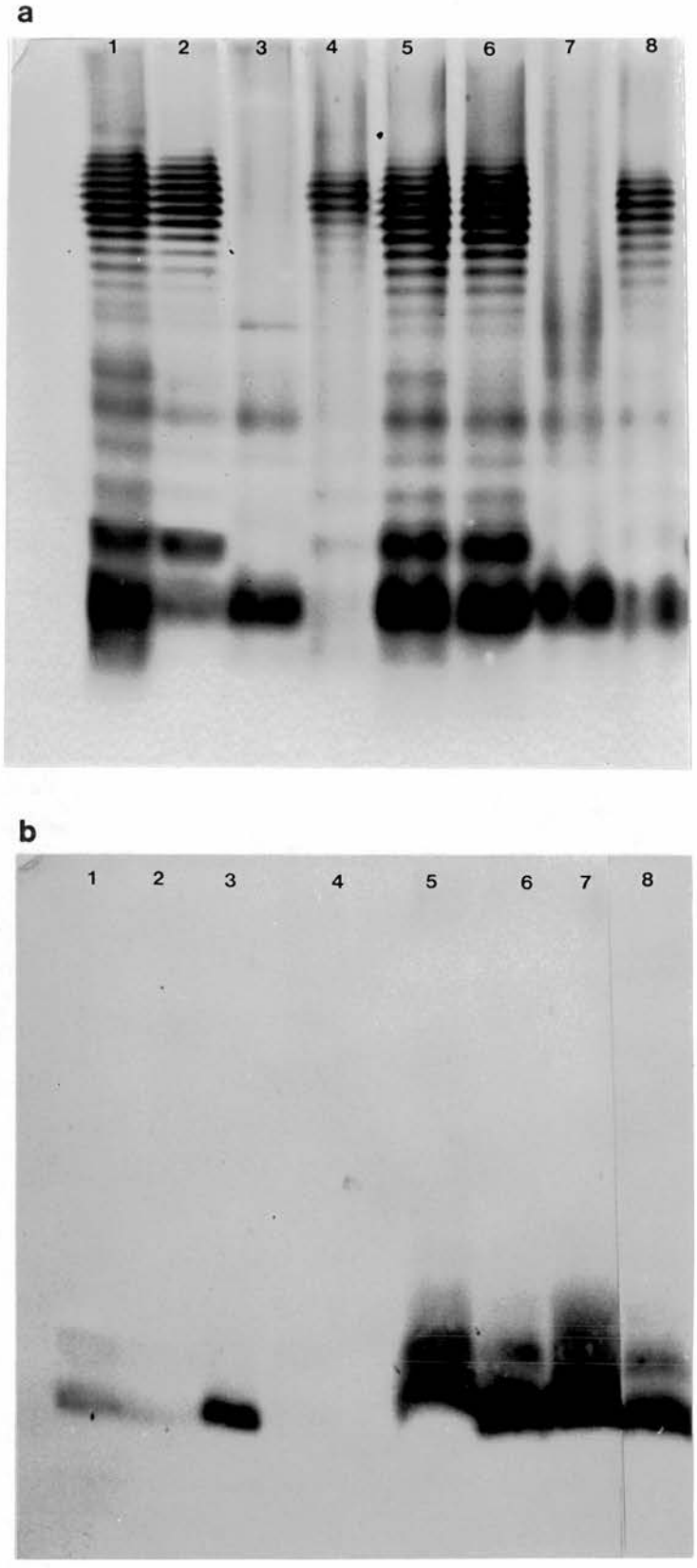


Figure 61. a) Silver stained LPS profiles of proteinase K whole cell digests of *E. coli* strains separated by PAGE (14% w/v acrylamide). b) Immunoblot following transfer of LPS to NIC paper and probing with a core-specific MAb, 43.27.11.2 Tracks 1-4 and 5-8 represent 4 *E. coli* strains (O18:K1, O18:K1 , O18:Krf and O6:K5) grown to early stationary phase in nutrient broth and a magnesium/iron depleted medium respectively.

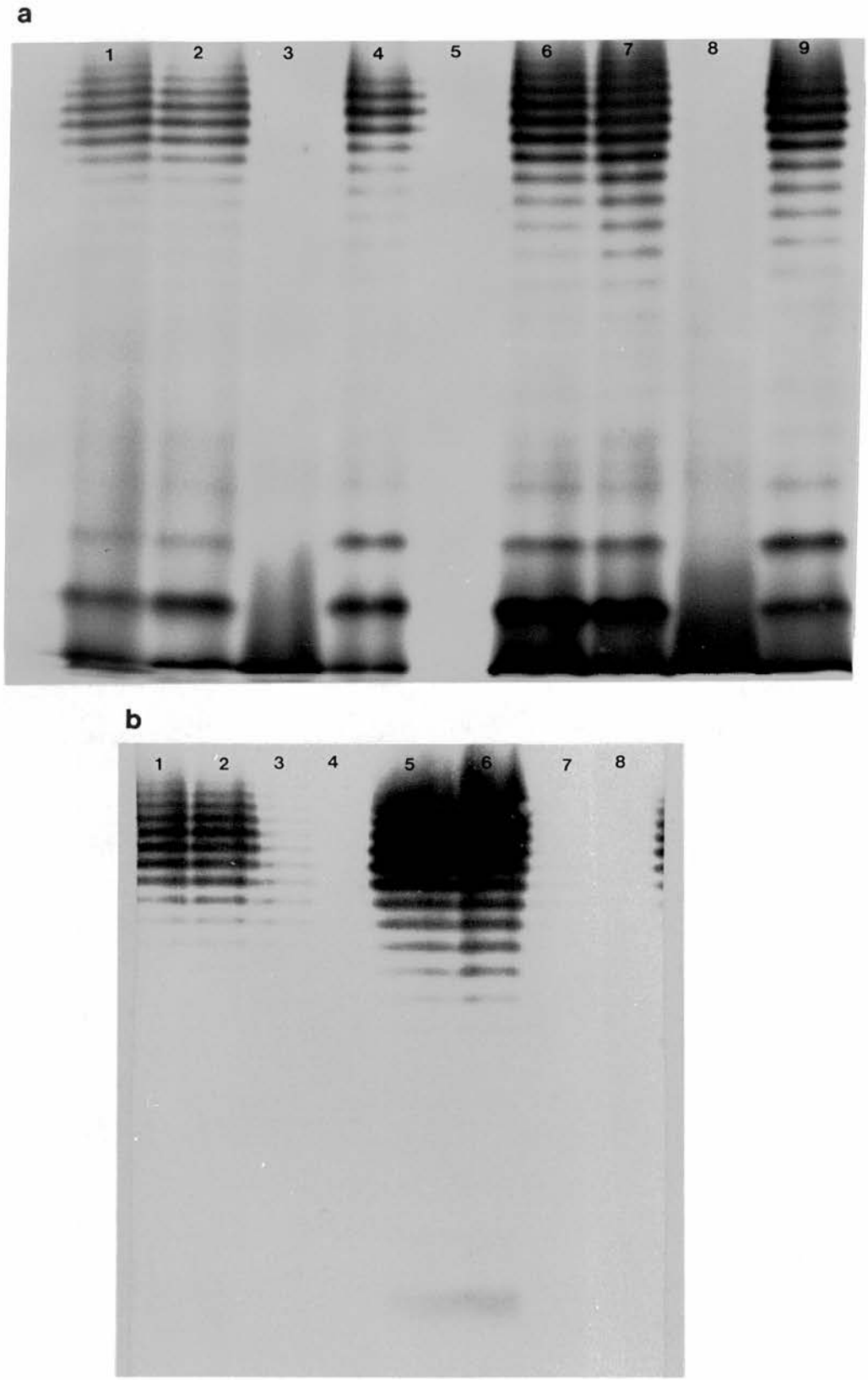


Figure 62. a) Silver stained LPS profiles of proteinase K whole cell digests of *E. coli* strains separated by PAGE (14% w/v acrylamide). b) Immunoblot following transfer of LPS to NIC paper and probing with an O18 O-antigen specific MAb, 184.2.5.5 Tracks 1-4 and 5-8 represent 4 *E. coli* strains (O18:K1, O18:K1$^-$, O18:Krf and O6:K5) grown to early stationary phase in nutrient broth and heat-inactivated sheep serum respectively.

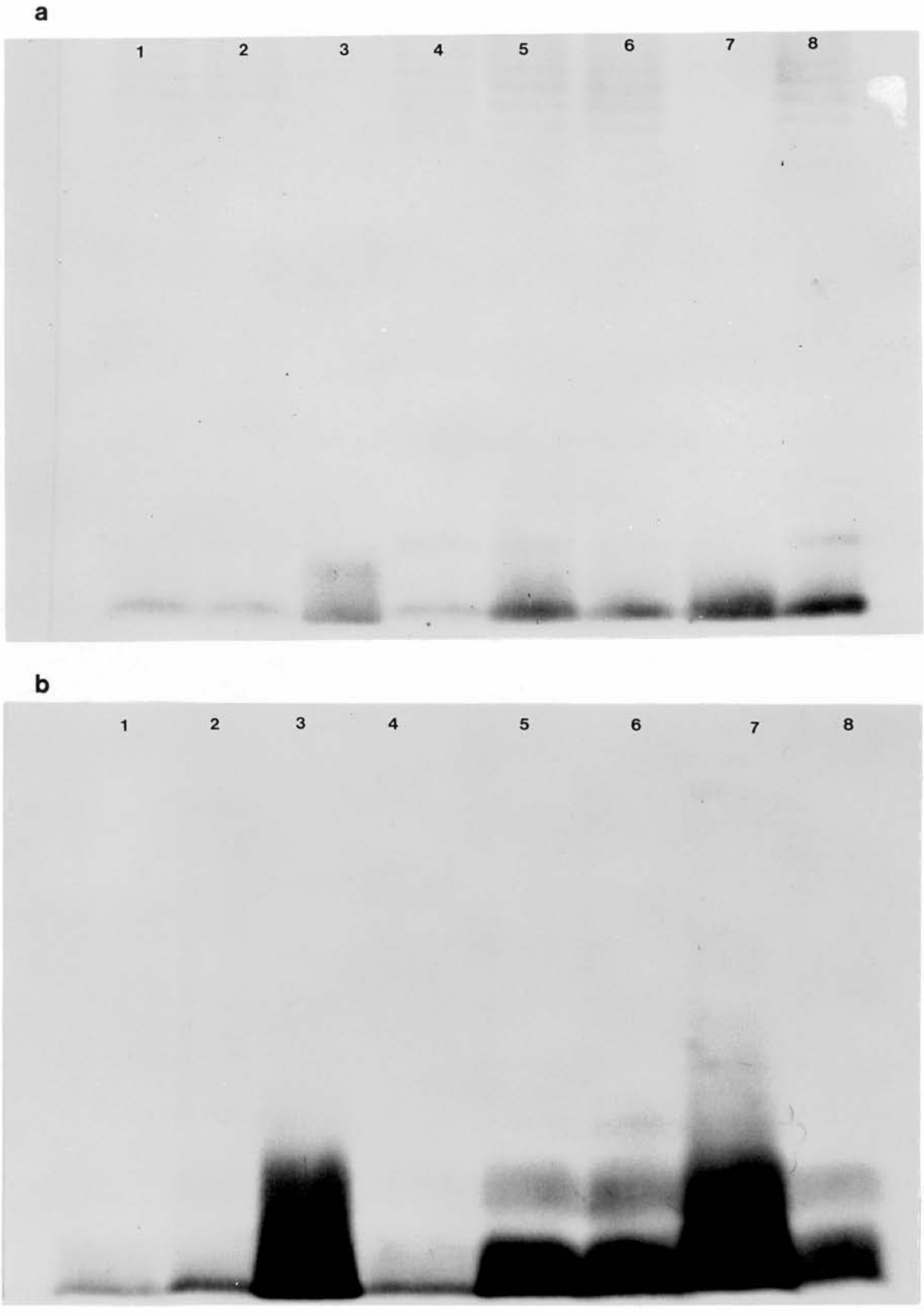


Figure 63. Immunoblots of proteinase K whole cell digests of *E. coli* strains separated by page (14% w/v acrylamide) followed by transfer to NIC paper and probed with two core-specific MAbs a) 27.150.3 and b) 43.27.11.2 Tracks 1-4 and 5-8 represent 4 *E. coli* strains (O18:K1, O18:K1 , O18:Krf and O6:K5) grown to early stationary phase in nutrient broth and heat-inactivated sheep serum respectively.

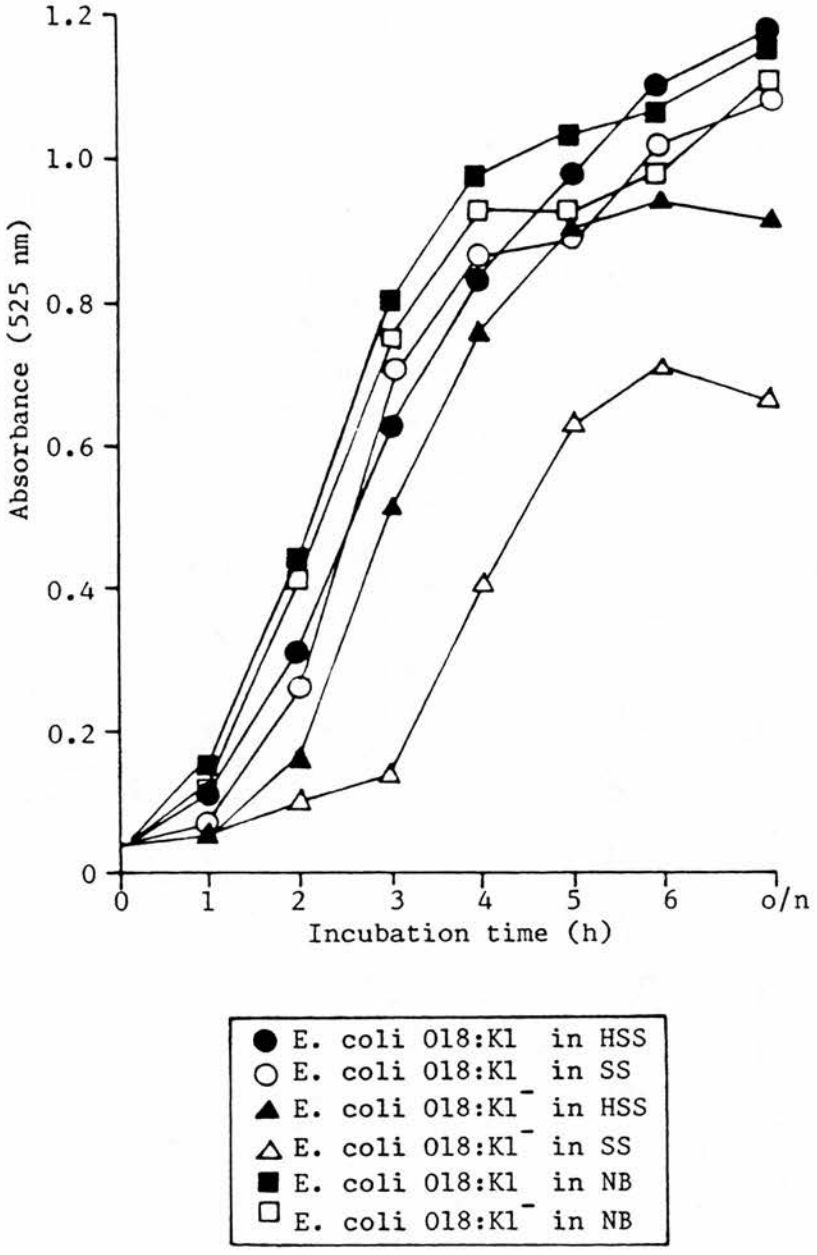


Figure 64. Growth of *E. coli* strains O18:K1 and O18:K1⁻ in nutrient broth (NB), heat-inactivated sheep serum (HSS) and untreated sheep serum (SS) as determined by measurement of absorbance of bacterial suspension at 525 nm.

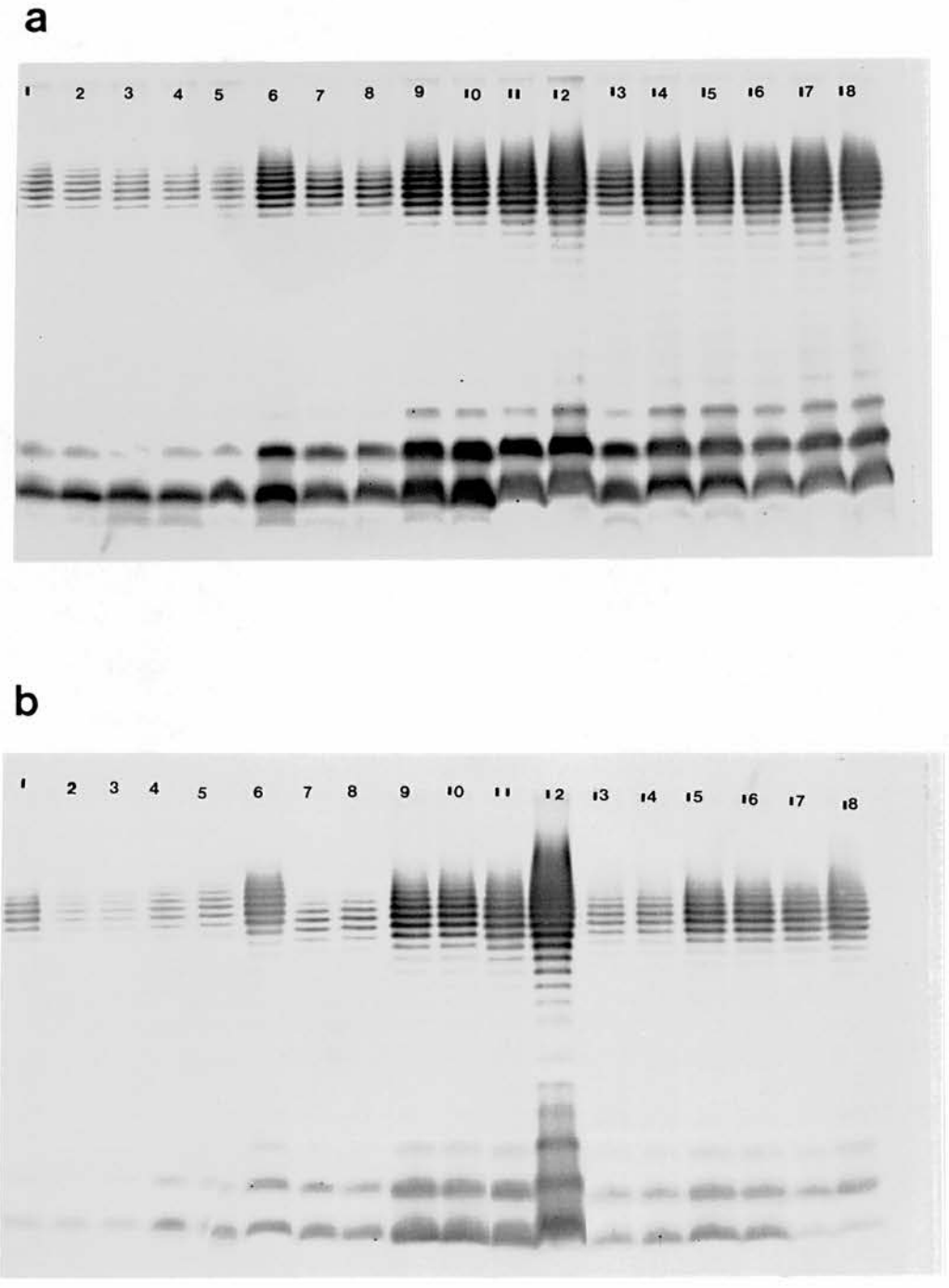


Figure 65. Silver stained LPS of proteinase K whole cell digests of a) *E. coli* O18:K1 and b) *E. coli* O18:K1 separated by page (14% w/v acrylamide). Tracks 1-6, 7-12 and 13-18 represent *E. coli* grown in nutrient broth, heat-inactivated sheep serum and untreated sheep serum respectively. Samples were removed at 1 h intervals from 2 h incubation to 6 h incubation, plus a sample from overnight incubation for each growth condition.

3.2 ELISA ON WHOLE BACTERIA GROWN *IN VITRO*

The binding activities of three anti-LPS MAbs to whole cells of four *E. coli* strains, cultured under the different growth conditions were investigated (Table 8). No significant differences in the binding of the O18, O-antigen specific MAb, 184.2.5.5 to O18:K1 and O18:Krf were detected for the different growth conditions. The O18 non-capsulate strain showed stronger affinity for both anti-core MAbs compared to its capsulate parent, when both were cultured under each growth condition. Growth of S-LPS *E. coli* strains in the nitrogen deficient/high carbon medium resulted in lower binding of the two anti-core MAbs compared to growth in nutrient broth. Significant increases in OD_{MAX} and D_{50} values were demonstrated for S-LPS organisms grown in heat-inactivated sheep serum and magnesium limitation (1%) and probed with anti-core MAbs 27.150.3 and 43.27.11.2. A similar, yet lower increase in binding of anti-core MAbs occurred for growth under iron depletion compared to nutrient broth.

The binding activities of two anti-core MAbs to *E. coli* O18:K1 grown in Malka minimal medium (17 mmol. L^{-1} Mg^{2+}) and modifications of this medium containing zero; 1% (0.17 mmol. L^{-1} Mg^{2+}); 5% (0.85 mmol. L^{-1} Mg^{2+}) and 10% (1.7 mmol. L^{-1} Mg^{2+}) volume of the magnesium salt used for the standard medium are shown in Figure 66. Increased binding of anti-core MAbs was observed for growth in all media containing reduced magnesium ion concentrations compared to the minimal medium. Maximal binding of MAbs occurred for cells grown in media containing no added magnesium salt or 0.17 mmol. L^{-1} (1%).

3.3 FLOW CYTOMETRIC ANALYSIS OF WHOLE BACTERIA GROWN *IN VITRO*

The effect of growth conditions on the expression of LPS on whole

Table 8. ELISA results of three anti-LPS MAbs titrated against whole cells of E. coli O18:K1, O18:K1⁻, O18:Krf and O6:K5 grown in five different media.

MAb	Growth Medium	ELISA Results							
		OD_{MAX}*				D_{50}**			
		E. coli				E. coli			
		O18:K1	O18:K1⁻	O18:Krf	O6:K5	O18:K1	O18:K1⁻	O18:Krf	O6:K5
184.2.5.5	Nutrient Broth	1.26	1.34	0.72	0	120	260	130	0
	Nitrogen Deficient/ High Carbon	1.31	1.33	0.69	0	145	280	200	0
	Iron Depleted	1.19	1.24	0.61	0	105	110	160	0
	Magnesium Depleted (1%)	1.39	1.19	0.72	0	120	75	110	0
	Serum	1.31	1.41	0.80	0	150	210	165	0
27.150.3	Nutrient Broth	0.45	0.51	1.37	0.23	40	40	310	35
	Nitrogen Deficient/ High Carbon	0.08	0.23	1.02	0.17	50	60	190	20
	Iron Depleted	0.62	0.98	1.70	0.53	56	36	360	60
	Magnesium Depleted (1%)	0.87	1.01	1.28	0.93	34	200	300	40
	Serum	0.73	1.24	1.54	1.04	270	370	370	95
43.27.11.2	Nutrient Broth	0.32	0.55	1.30	0.36	37	36	145	40
	Nitrogen Deficient/ High Carbon	0.17	0.20	1.21	0.22	23	40	115	35
	Iron Depleted	0.65	0.82	1.28	0.51	32	90	145	30
	Magnesium Depleted (1%)	0.88	1.18	1.35	0.76	66	120	150	145
	Serum	0.97	1.15	1.55	0.79	140	180	160	150

* = Maximum OD_{590} for the 1:10 dilution (first dilution) of antibody.

** = The dilution factor when OD is 50% of the maximum.

The results are calculated from the means of three experiments each carried out in triplicate.

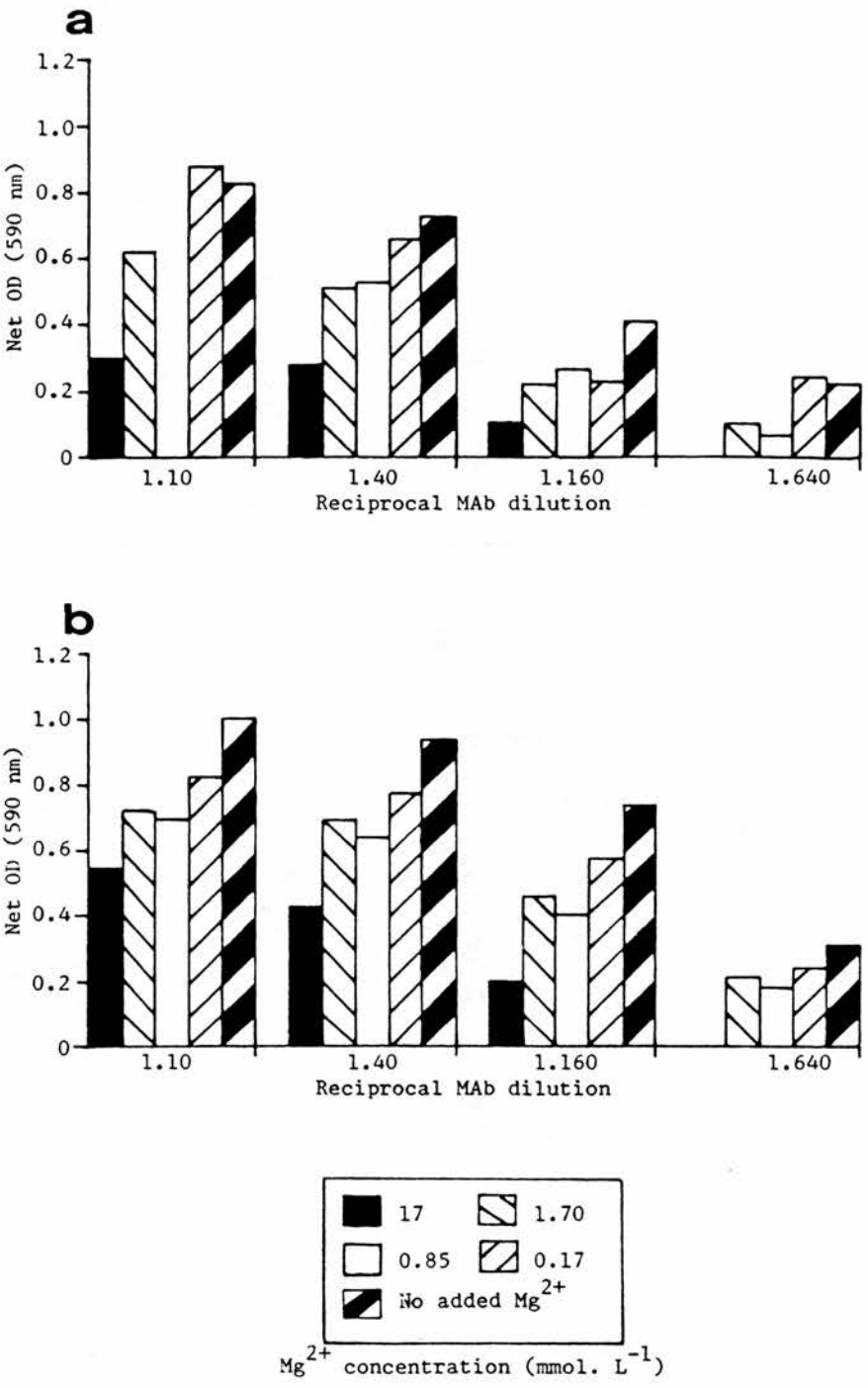
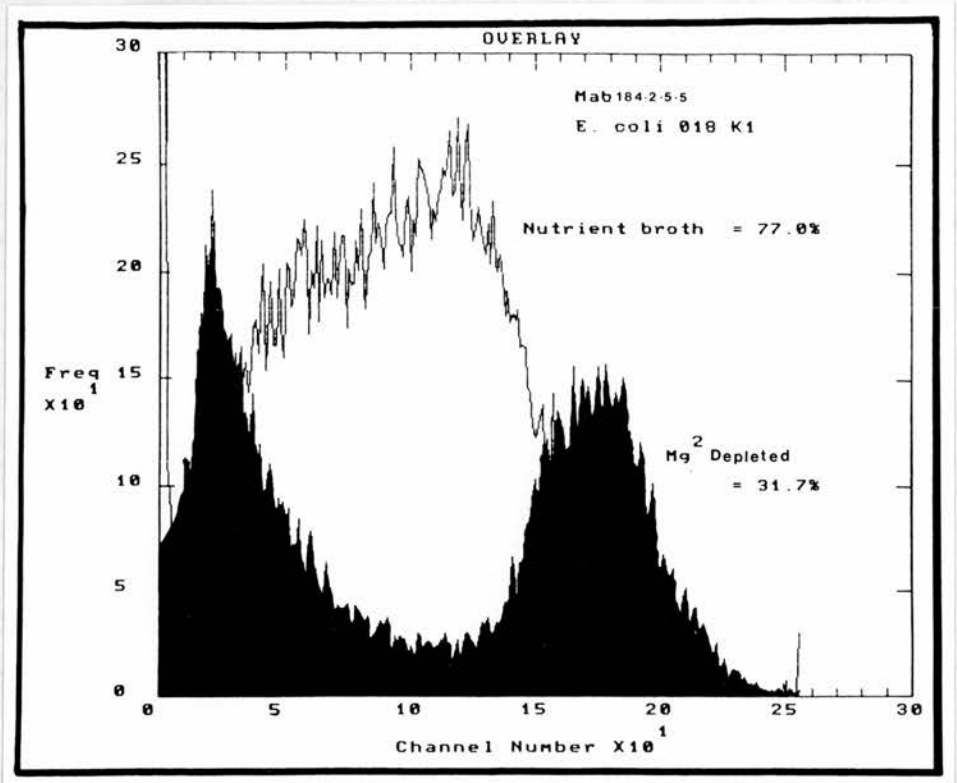


Figure 66. ELISA activity of anti-core LPS MAbs 43.27.11.2 (a) and 43.3.4.8 (b) against *E. coli* O18:K1 grown to early stationary phase in Malka minimal medium (17 mmol. L^{-1} Mg^{2+}), or modifications of this medium containing 10% (1.70 mmol. L^{-1} Mg^{2+}); 5% (0.85 mmol. L^{-1} Mg^{2+}); 1% (0.17 mmol. L^{-1} Mg^{2+}) and zero volume of the magnesium salt used for the Malka minimal medium. Each histogram bar represents the mean optical density value of three separate experiments carried out in triplicate.

bacteria was also investigated by flow cytometry. Flow cytometric profiles, which relate to the intensity of fluorescence signal (ie antibody binding to bacteria), were obtained consistently in three separate experiments. Examples of representative profiles are illustrated in Figures 67 and 68. The histograms produced by the interaction of the O18, O-antigen specific MAb, 184.2.5.5, and *E. coli* O18:K1 grown in nutrient broth and a magnesium depleted (1%) medium are shown in Figure 67a. When compared with nutrient broth cells a 45% decrease in magnesium depleted bacteria exhibiting positive fluorescence above background levels was observed. The biphasic fluorescence pattern produced by magnesium depleted cells demonstrated the presence of two distinct subpopulations of *E. coli* O18:K1 on the basis of differential binding of 184.2.5.5. Probing the same cells with core-specific MAb 43.27.11.2 resulted in a significant increase in positive fluorescence compared to nutrient broth grown cells (Figure 67b). Significant increases in the binding levels of both anti-core MAbs 43.27.11.2 and 27.150.3 were also observed when *E. coli* O18:K1$^-$ was grown in heat-inactivated serum compared to nutrient broth (Figure 68).

Flow cytometry data, showing the effect of growing four *E. coli* strains under the different growth conditions on MAb recognition of LPS associated epitopes is presented in Tables 9 & 10. Although relative differences between growth conditions remained constant, day to day variations in percentage labelling within a given sample was evident. Probing cells with O18, O-antigen specific MAb 184.2.5.5, resulted in similar binding levels for most conditions, whilst magnesium depleted (1%) cells, notably O18:K1$^-$ showed a significant decrease. Smooth cells grown under heat-inactivated sheep serum and magnesium (1%) conditions again showed the largest increases in binding of anti-core MAbs.

a



b

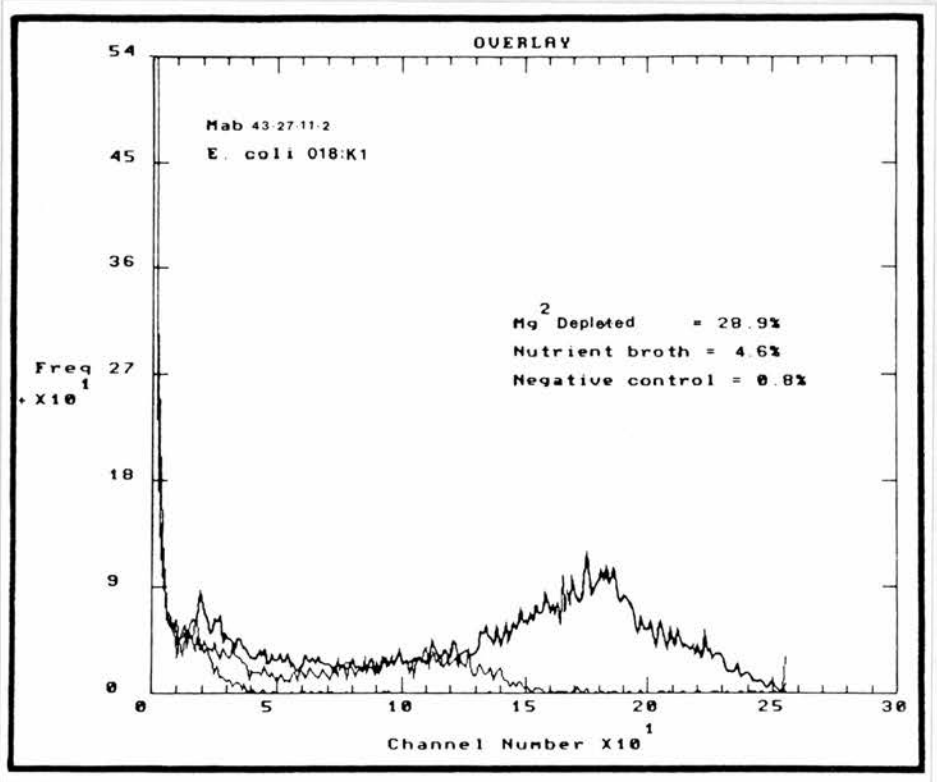
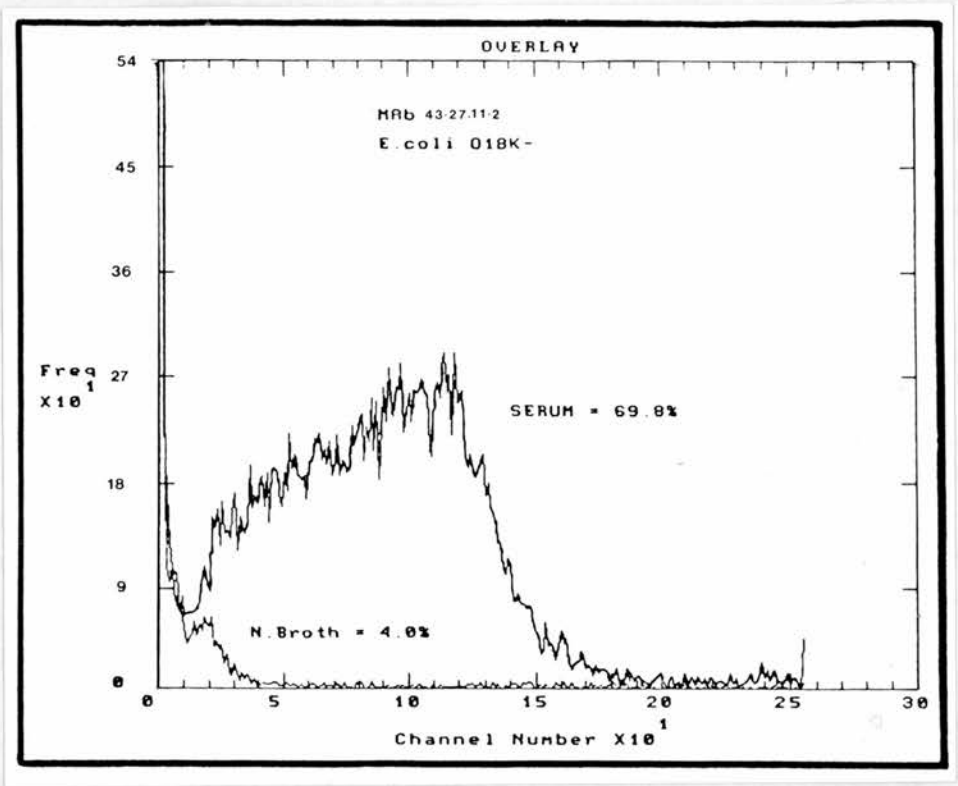


Figure 67. Green fluorescence intensity histograms of two anti-LPS MAbs against whole cells of O18:K1 grown to early stationary phase in nutrient broth and a magnesium depleted (1%) medium. a) represents the binding of an O18 O-antigen specific MAb, 184.2.5.5 and b) a core-specific MAb, 43.27.11.2 Percentage values represent bacteria exhibiting positive fluorescence above background levels.

a



b

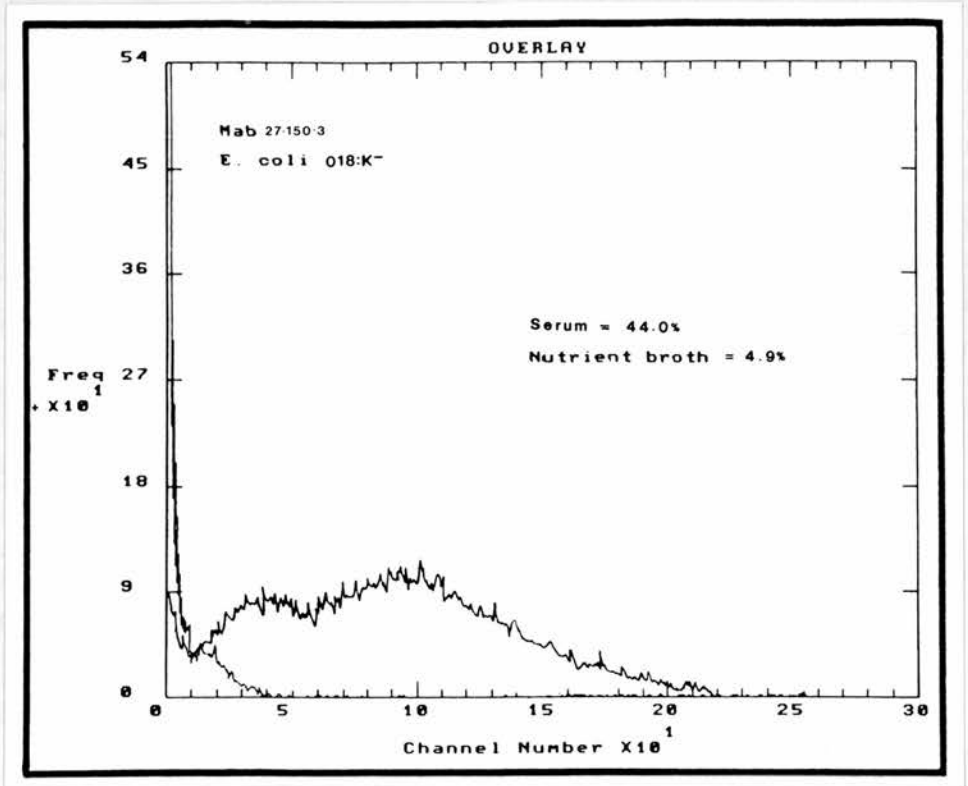


Figure 68. Green fluorescence intensity histograms of two anti-LPS MAbs against whole cells of O18:K1 grown to early stationary phase in nutrient broth and heat-inactivated sheep serum. (a & b) represent the binding of two core specific MAbs 43.27.11.2 and 27.150.3 respectively. Percentage values represent bacteria exhibiting positive fluorescence above background levels.

Table 9. Flow cytometric analysis of anti-LPS MAb binding to whole cells of 4 *E. coli* strains grown under different conditions. Percentage values represent the mean positive fluorescence of bacteria above background levels from three separate experiments. MAbs include O18, O-antigen specific MAb 184.2.5.5, and core- specific MAb 27.150.3.

		Percentage of cells showing positive fluorescence*			
		E. coli			
MAb	Growth Medium	O18:K1	O18:K1$^-$	O18:Krf	O6:K5
184.2.5.5	Nutrient Broth	70(19)	62(14)	2 (1)	ND
	Nitrogen Deficient/ High Carbon	73(10)	68 (7)	13 (6)	ND
	Iron Depleted	74 (6)	50 (3)	3 (1)	ND
	Magnesium Depleted (1%)	49 (9)	39 (9)	7 (0)	ND
	Serum	68(15)	67 (8)	8 (1)	ND
27.150.3	Nutrient Broth	2 (1)	6 (1)	26(11)	3(2)
	Nitrogen Deficient/ High Carbon	2 (0)	3 (1)	16 (6)	2(1)
	Iron Depleted	3 (2)	8 (3)	20 (5)	14(9)
	Magnesium Depleted (1%)	12 (4)	18 (2)	28 (7)	10(2)
	Serum	16 (5)	34(11)	20(12)	32(8)

* = Mean (±SD)

ND = Not Done

Table 10. Flow cytometric analysis of anti-LPS MAb binding to whole cells of 4 *E. coli* strains grown under different conditions. Percentage values represent the mean positive fluorescence of bacteria above background levels from three separate experiments. MAbs include core-specific MAbs 43.27.11.2 and 43.3.4.8.

		Percentage of cells showing positive fluorescence*			
		E. coli			
MAb	Growth Medium	O18:K1	O18:K1$^-$	O18:Krf	O6:K5
	Nutrient Broth	3 (1)	3 (2)	68(11)	7 (1)
	Nitrogen Deficient/ High Carbon	2 (0)	2 (1)	56(12)	1 (0)
43.27.11.2	Iron Depleted	10 (7)	7 (4)	66 (7)	7 (3)
	Magnesium Depleted	21 (4)	24 (5)	75(13)	18 (8)
	Serum	28(16)	61(11)	43(14)	26(12)
	Nutrient Broth	32 (8)	38 (5)	81(14)	29 (4)
	Nitrogen Deficient/ High Carbon	6 (1)	14 (8)	77(11)	8 (2)
43.3.4.8	Iron Depleted	38 (4)	51(11)	87 (8)	41 (7)
	Magnesium Depleted (1%)	42 (9)	48 (6)	79 (9)	49 (9)
	Serum	52 (6)	67(10)	84(12)	54 (5)

* = Mean (±SD)

ND = Not Done

Table 11. Flow cytometric analysis of anti-LPS MAb binding to whole cells of *E. coli* strains O18:K1 and O18:K1$^-$ grown in absorbed and non-absorbed heat-inactivated sheep serum. Percentage values represent the mean positive fluorescence of bacteria above background levels from triplicate readings. MAbs include core-specific MAb 43.27.11.2 and O18, O-antigen specific MAb 184.2.5.5.

	Percentage of cells showing positive fluorescence*			
	Mab 43.27.11.2		MAb 184.2.5.5	
	E. coli		*E. coli*	
Absorbant	O18:K1	O18:K1$^-$	O18:K1	O18:K1$^-$
None	31 (6)	55 (8)	79 (8)	73 (9)
E. coli O18:K1$^-$	24(12)	57(10)	83(12)	69 (7)
E. coli O6:K5	29 (8)	49 (5)	71 (5)	65(12)

* = Mean (±SD)

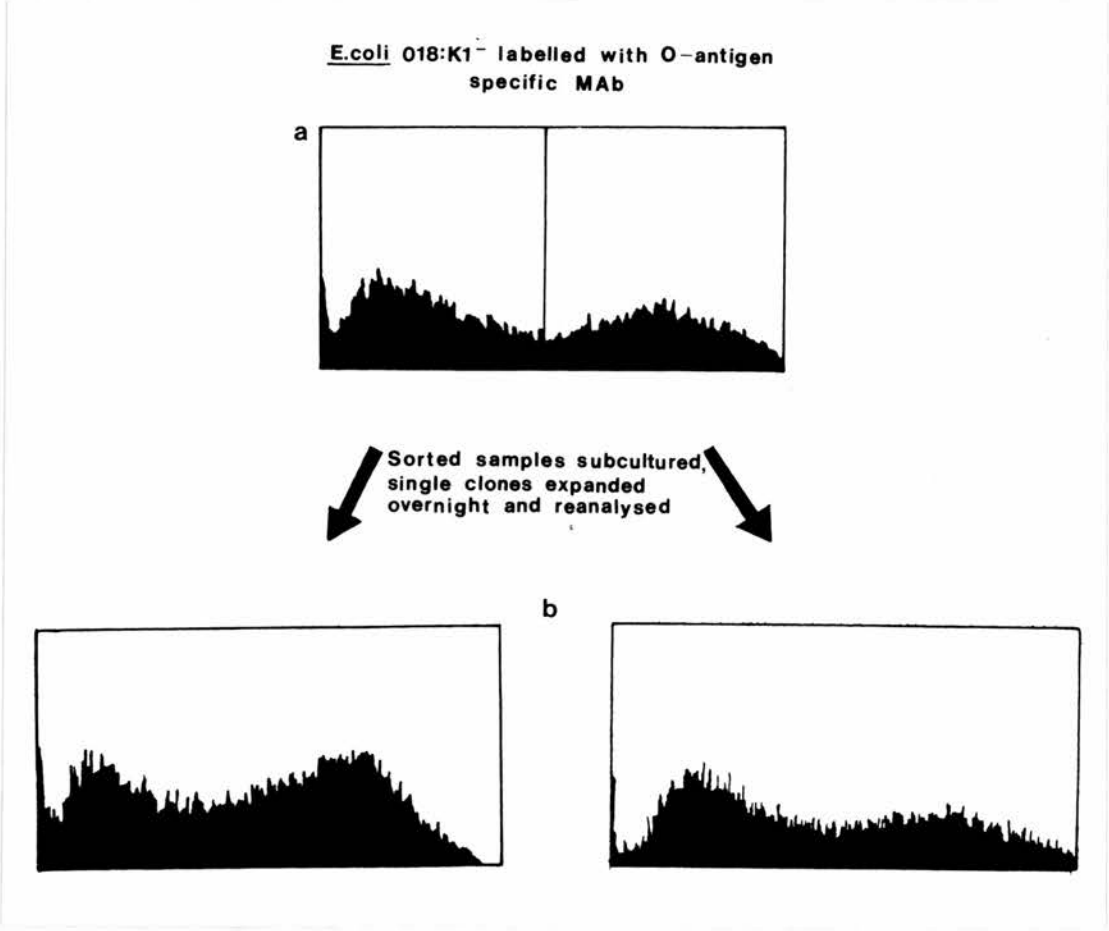


Figure 69. Subpopulations of *E. coli* 018:K1⁻ identified and sorted by flow cytometry on the basis of differential binding by O-antigen specific MAb, 184,2.5.5. Green fluorescence intensity histograms represent presorted (a) and postsorted (b) cell populations.

Nitrogen deficient/high carbon cells showed an overall decrease in percentage labelling when probed with anti-core MAbs, especially 43.3.4.8. Overall, greater binding of anti-core MAbs was demonstrated against the rough mutant O18:Krf compared to smooth strains.

The binding activities of anti-LPS MAbs to *E. coli* O18:K1 and O18:K1$^-$ grown in absorbed and unabsorbed heat-inactivated sheep serum was compared (Table 11). Absorption of serum was performed with organisms possessing an identical or unrelated O-antigenic structure to that under analysis. Growth of each organism in unabsorbed or absorbed serum produced similar positive fluorescence values when probed with either core or O-antigen specific MAbs. The anti-core MAb again demonstrated greater reactivity to the non-capsulate strain.

The feasibility of sorting viable bacteria on the basis of differential fluorescence signals produced by surface-bound MAb was investigated. An example of biphasic fluorescent labelling representing two distinct subpopulations of cells was illustrated by the differential binding of O-antigen reactive MAb 184.2.5.5 to *E. coli* O18:K1$^-$ grown in nutrient broth. The two subpopulations were sorted into nutrient broth, expanded and reanalysed by flow cytometry (Figure 69). Analysis of the subcultured cells from both subpopulations following staining by MAb 184.2.5.5, revealed that they were unstable - each producing both subpopulations.

3.4 IMMUNOGOLD ELECTRON MICROSCOPY OF BACTERIA GROWN *IN VITRO*

Immunogold electron microscopy was also used to assess the binding of anti-LPS MAbs to *E. coli* cells grown under selected nutrient conditions and processed for thin-sections. Figure 70a-c shows the binding of O-

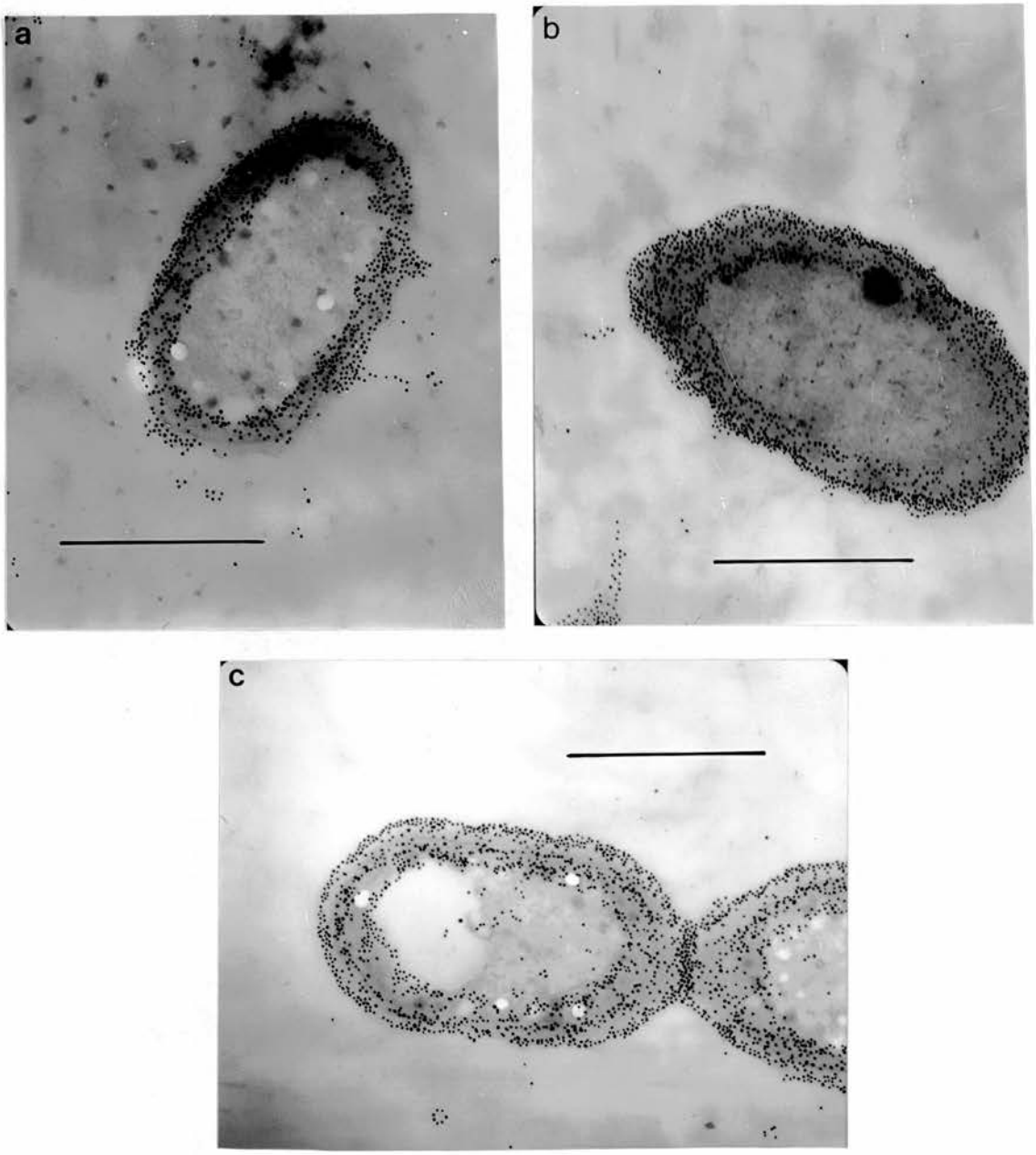


Figure 70. Immunogold electron micrographs of thin sections from cells of *E. coli* O18:K1 grown in nutrient broth (a), nitrogen deficient/high carbon medium (b) and heat-inactivated sheep serum (c). Cells were prepared by probing with O-antigen specific MAb 184.2.5.5 followed by anti-mouse IgG gold conjugate. Bars, 1 μm.

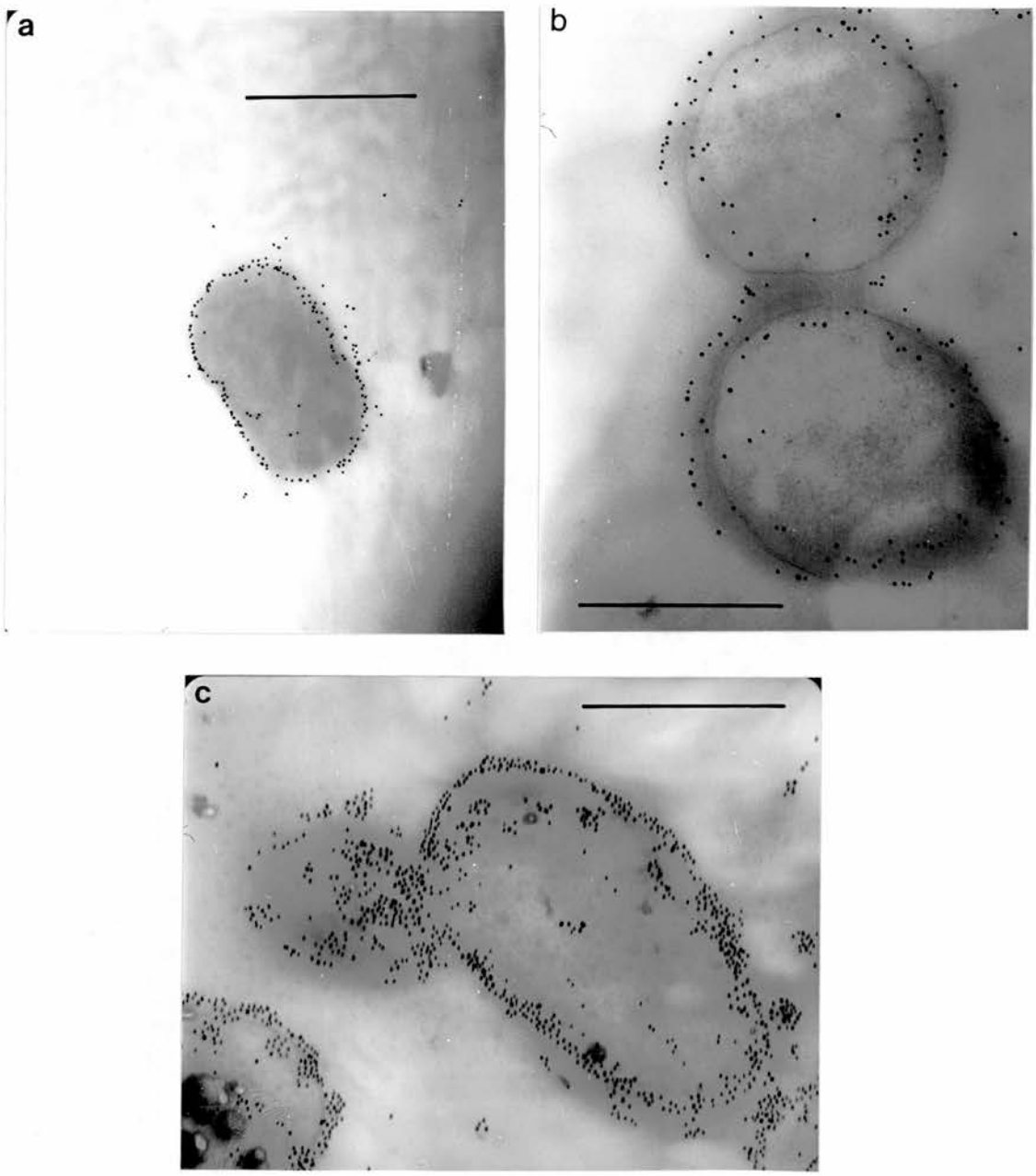


Figure 71. Immunogold electron micrographs of thin sections from cells of *E. coli* O18:K1 grown in nutrient broth (a), nitrogen deficient/high carbon medium (b) and heat-inactivated sheep serum (c). Cells were prepared by probing with core specific MAb 27.150.3, followed by anti-mouse IgG gold conjugate. Bars, 1 μm.

antigen specific MAb 184.2.5.5 to representative *E. coli* O18:K1 cells grown in nutrient broth, nitrogen deficient/high carbon medium and heat-inactivated sheep serum. Immunolabelling of the sections resulted in extensive gold labelling of the surface of the cells. Figure 71a-c illustrates representative sections of cells grown under identical conditions to those described above, and probed with a core-specific MAb 27.150.3. Growth of *E. coli* O18:K1 in heat-inactivated sheep serum showed the greatest MAb binding (c), whilst growth in nitrogen deficient/high carbon medium showed the least (b).

3.5 LIPOPOLYSACCHARIDE ANALYSIS OF *IN VIVO* GROWN BACTERIA

The expression of LPS on *in vivo* grown bacteria was investigated using two animal models: i) chambers implanted in the mouse peritoneal cavity and ii) the mucin-haematin septicaemia model.

Chamber implants were removed after 2 days, at which stage the bacterial cultures had reached their maximal density of 10^8-10^9 cells ml^{-1}. The silver stained LPS profiles of *in vitro* nutrient broth and *in vivo* chamber implant grown *E. coli* O18:K1 are shown in Figure 72. Compared with the *in vitro* cells, *in vivo* cells appeared to express greater amounts of O-antigen as well as an overall increase in O-polysaccharide chain length. *In vivo* LPS profiles also showed greater expression of unsubstituted core material and other low molecular mass bands.

Silver staining and immunoblot analysis were used to investigate the LPS profiles of *E. coli* O18:K1 grown *in vivo* in chamber implants, and recovered from the mouse peritoneal cavity 1-10 days after implantation. Silver stained LPS showed no significant differences between

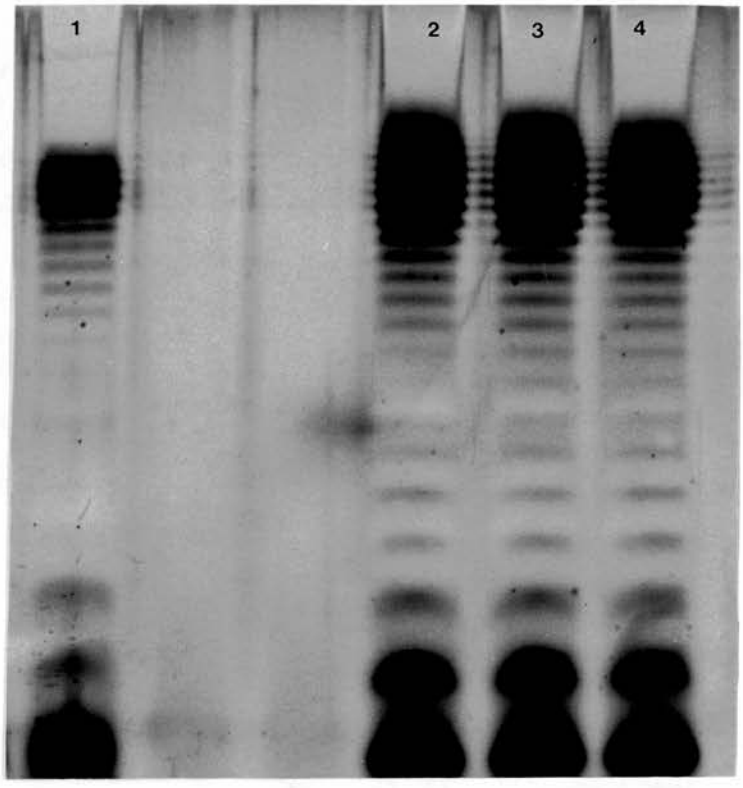


Figure 72. Silver stained LPS profiles of proteinase K whole cell digests of *E. coli* O18:K1 separated by PAGE (14% w/v acrylamide). Track 1, cells grown *in vitro* in nutrient broth, and Tracks 2-4, cells grown *in vivo* in mouse chamber implants. Tracks 2-4 represent cell digests prepared from chambers taken from three separate mice 2 days after implantation.

incubation times (Figure 73). However, cells recovered between 2 and 10 days appear to express greater high molecular mass O-antigenic material compared to cells recovered after 1 day (Track 1). Corresponding immunoblots probed with O18, O-antigen specific MAb 184.2.5.5 and core-specific MAb 43.27.11.2 are shown in Figure 74. Serotype specificity of *E. coli* O18:K1 was retained when the organisms were grown *in vivo*. Reactivity of MAb 184.2.5.5 was greatest against the LPS of cells recovered once 4 days implantation had elapsed. MAb 43.27.11.2 reacted weakly against samples recovered following an incubation period of 1 day (Track 1) and 3 days (Track 3). The strongest reactions were against samples recovered following an incubation period of 4 days (Track 4) and 10 days (Track 6).

The mucin-haematin septicaemia model was used for the *in vivo* growth of *E. coli* O18:K1 and O16:K1 in mice. Bacteria were recovered from whole blood and subjected to silver staining analysis (Figure 75). The LPS profiles of the two *in vivo* grown *E. coli* O18:K1 samples were shown to contain longer O-polysaccharide chain lengths than *in vitro* grown cells. The O-polysaccharide chain lengths of the two *in vivo* grown *E. coli* O16:K1 samples were shown to be either the same (Track 2) or longer (Track 3) than cells grown *in vitro* (Track 1).

Preliminary ELISA investigations compared the binding activities of anti-LPS MAbs *in vitro* or *in vivo* grown whole bacterial cells coated directly to the wells of microtitre plates. Results established high background signals of wells coated with *in vivo* grown cells from chamber implants or whole blood. Further studies compared the ability of *in vitro* and *in vivo* whole bacterial cells to inhibit the ELISA reactivity of anti-LPS MAbs against coated *in vitro* grown whole cells.

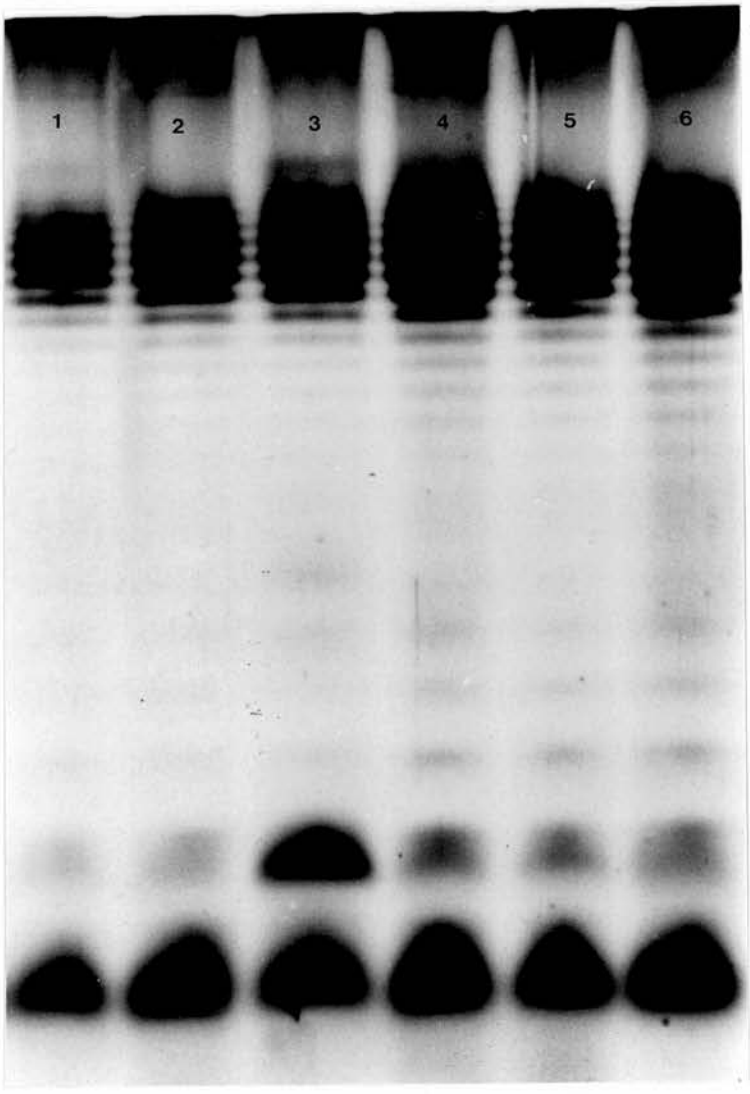


Figure 73. Longitudinal study of silver stained LPS profiles of proteinase K whole cell digests of *in vivo* grown *E. coli* O18:K1 separated by PAGE (14% w/v acrylamide). Chamber implants were recovered from mice 1 day (Track 1); 2 days (Track 2); 3 days (Track 3); 4 days (Track 4); 7 days (Track 5) and 10 days (Track 6) after implantation.

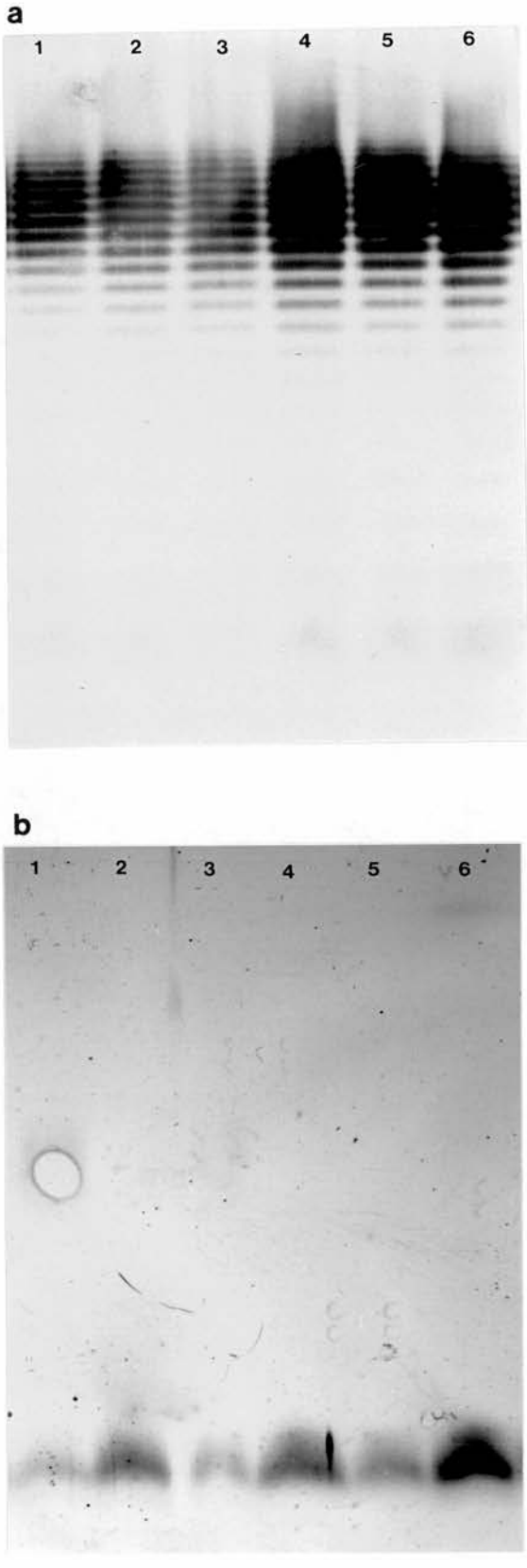


Figure 74. Immunoblots of *in vivo* grown proteinase K whole cell digests of *E. coli* O18:K1 separated by PAGE (14% w/v acrylamide) followed by transfer to NIC paper and probed with a) O18, O-antigen specific MAb 184.2.5.5 and b) core-specific MAb 43.27.11.2. Chamber implants were recovered from mice 1 day (Track 1); 2 days (Track 2); 3 days (Track 3); 4 days (Track 4); 7 days (Track 5) and 10 days (Track 6) after implantation.

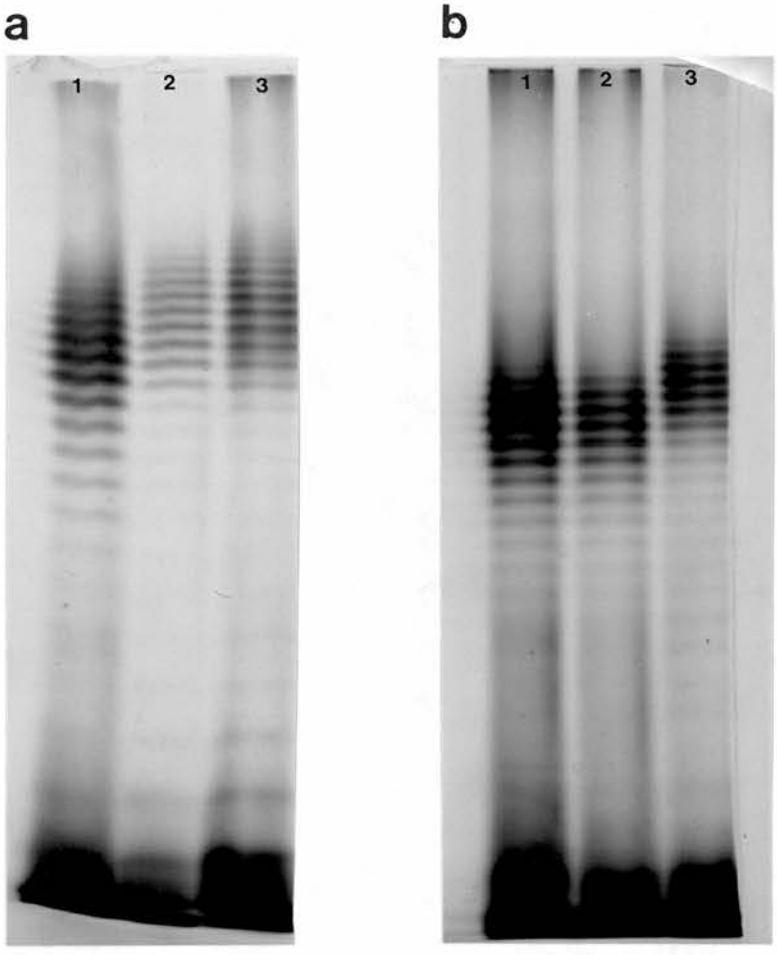


Figure 75. Silver stained LPS profiles of proteinase K whole cell digests of 2 *E. coli* strains, *E. coli* 018 (a) and *E. coli* 016 (b) separated by PAGE (14% w/v acrylamide). For each strain, Track 1 represents cells grown *in vitro* in nutrient broth and Tracks 2 and 3, cells grown *in vivo* in a mouse septicaemia model. Tracks 2 and 3 represent cells recovered from 2 separate mice.

E. coli O18:K1 grown in either nutrient broth or chambers implanted in the mouse peritoneal cavity were prepared in a ten-fold dilution series from $1x10^9$ cells ml^{-1}. All inhibitor dilutions were mixed with an equal volume of MAb diluted in dilution buffer (184.2.5.5 1:40, 43.27.11.2 1:10), and preincubated at 37°C for 30 min before adding to ELISA. Both *in vitro* and *in vivo* cell preparations demonstrated a similar inhibitory activity of O-specific MAb 184.2.5.5 (Figure 76a). Binding of the MAb was completely inhibited by both cell preparations at an inhibitor concentration of $1x10^9$ cells ml^{-1}. Overall, the inhibitory activity of *in vivo* grown cells was slightly greater than *in vitro* grown cells. The corresponding inhibition of anti-core MAb 43.27.11.2 is shown in Figure 76b. For each inhibitor dilution, *in vivo* cells showed greater inhibition of ELISA activity compared to *in vitro* cells. At an inhibitor concentration of $1x10^9$ cells ml^{-1} residual ELISA activity was 18% for *in vivo* cells and 52% for *in vitro* cells.

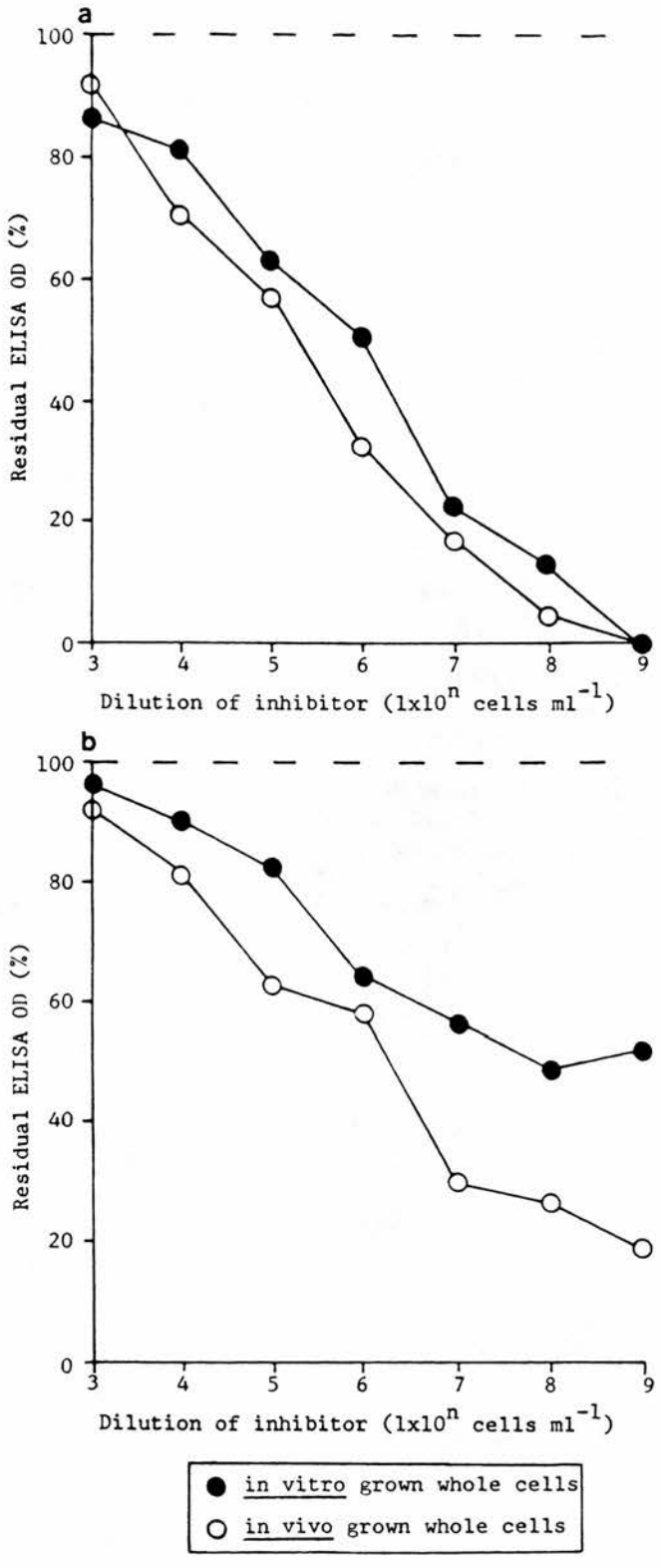


Figure 76. Inhibition of ELISA reactivity of O-specific MAb 184.2.5.5 (1:40) (a) and core-specific MAb 43.27.11.2 (1:10) (b) against *in vitro* nutrient broth grown *E. coli* 018:K1 whole cells with *in vitro* nutrient broth grown *E. coli* 018:K1 whole cells and *in vivo* (mouse chamber implants) grown *E. coli* 018:K1 whole cells. Inhibitors were diluted from $1x10^9$ cells ml^{-1} to $1x10^3$ cells ml^{-1}. Residual ELISA reactivity in the presence of inhibitor is expressed as a percentage of reactivity with no inhibitor (the 100% value, represented by the dotted line). Results represent the means of triplicate readings.

CHAPTER 4

THE EFFECTS OF SUBINHIBITORY CONCENTRATIONS OF ANTIBIOTICS ON THE BINDING OF ANTI-LIPOPOLYSACCHARIDE MONOCLONAL ANTIBODIES TO *ESCHERICHIA COLI*

The binding capacity of anti-LPS MAbs to *E. coli* bacteria grown in both the absence and presence of sub-MICs of various antibiotics was studied. The effect of sub-MICs of antibiotics on the expression of isolated LPS and outer membrane proteins was also investigated. Eight *E. coli* strains (six clinical blood culture isolates and two isogenic mutants of the O18:K1 parent: a non-capsulate mutant, O18:K1$^-$, and a rough mutant, O18:Krf) were grown in the absence and presence of the β-lactam antibiotic ampicillin; chloramphenicol; the aminoglycoside gentamicin and the fluoroquinolone ciprofloxacin. The MICs of each antibiotic for the *E. coli* strains are shown in Table 12.

4.1 SILVER STAINING AND IMMUNOBLOT ANALYSIS OF LIPOPOLYSACCHARIDE FROM ANTIBIOTIC TREATED AND UNTREATED CELLS

The silver stained LPS profiles of eight *E. coli* strains grown in the absence and presence of sub-MICs of ampicillin, chloramphenicol and gentamicin are shown in Figures 77 and 78. Major visible changes in LPS expression were only observed for most of the strains cultured in the presence of chloramphenicol. Changes included a decreased expression of high molecular mass, O-antigen bearing LPS bands (for example, Figure 77b & c, Tracks 5-7), and/or an increased expression of low molecular mass substituted LPS bands (for example, Figure 78b, Tracks 5-7). These changes were more prominent for the lowest sub-MIC dilution (1:2). Immunoblotting LPS profiles of *E. coli* O18:K1 with

Table 12. MICs of ampicillin, chloramphenicol, gentamicin and ciprofloxacin for *E. coli*.

	MIC (μg/ml)			
Strain	Ampicillin	Chloramphenicol	Gentamicin	Ciprofloxacin
O18:K5 (226)	4	16	0.5	0.032
O18:K5 (281)	128	8	0.5	0.008
O1:K1 (316)	2	8	0.5	0.016
O6:K? (317)	256	16	0.5	0.032
O?:K5 (484)	2	16	0.5	0.016
O18:K1	2	8	0.5	0.016
O18:K1$^-$ (isogenic mutant of above)	2	8	0.5	0.016
O18:Krf (isogenic mutant of above)	2	8	0.5	0.008

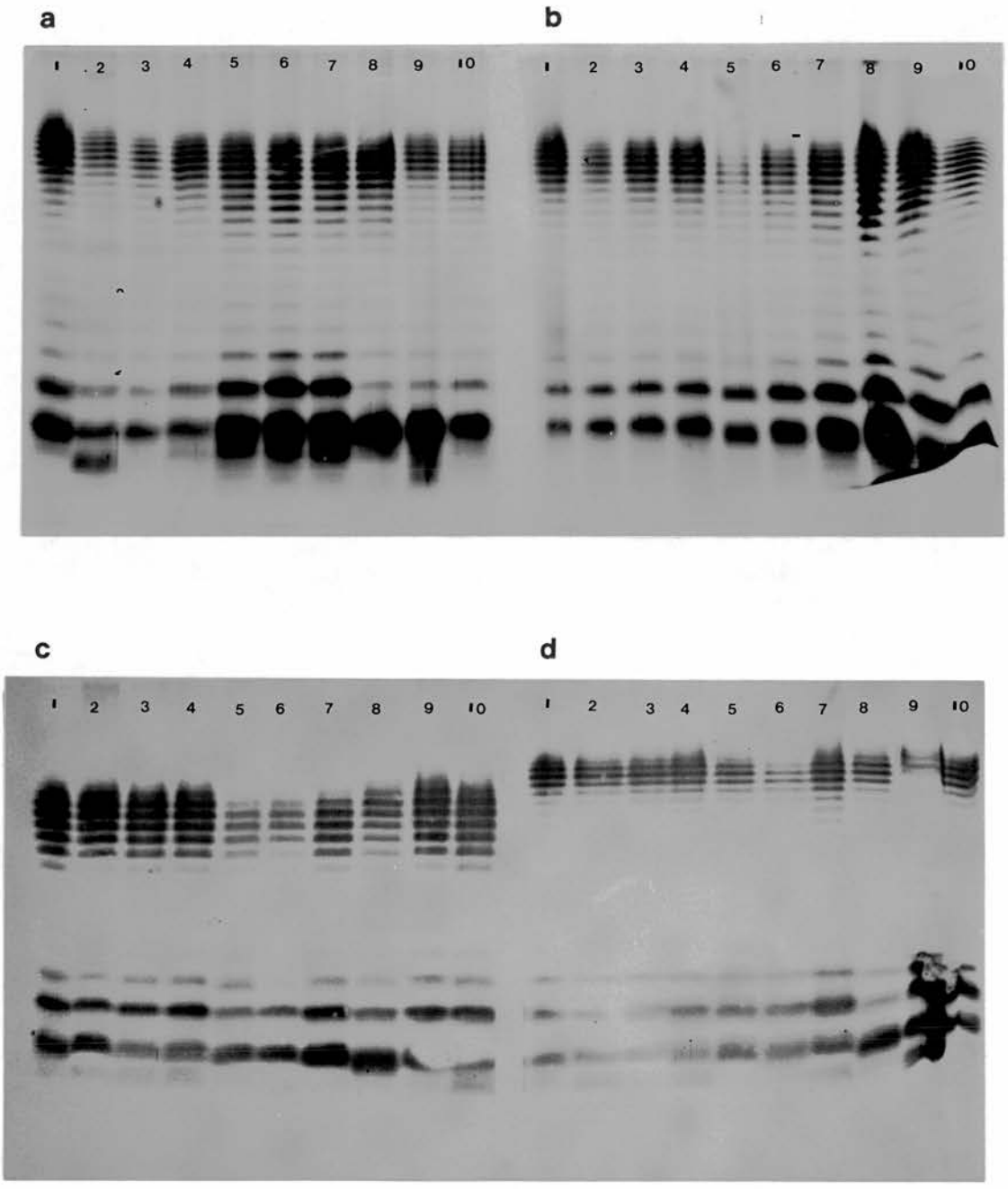


Figure 77. Silver stained LPS profiles of proteinase K whole cell digests of *E. coli* strains O18:K5 (226) (a); O18:K5 (281) (b); O1:K1 (316) (c) and O6:K? (317) (d) separated by PAGE (14% w/v acrylamide). For each strain, Track 1 represents cells grown in the absence of antibiotic and Tracks 2-4, 5-7 and 8-10 represent cells grown in the presence of 1:2, 1:4 and 1:8 MIC dilutions of ampicillin, chloramphenicol and gentamicin respectively.

NA = no antibiotic, Amp = ampicillin, Chlor = chloramphenicol and Gent = gentamicin.

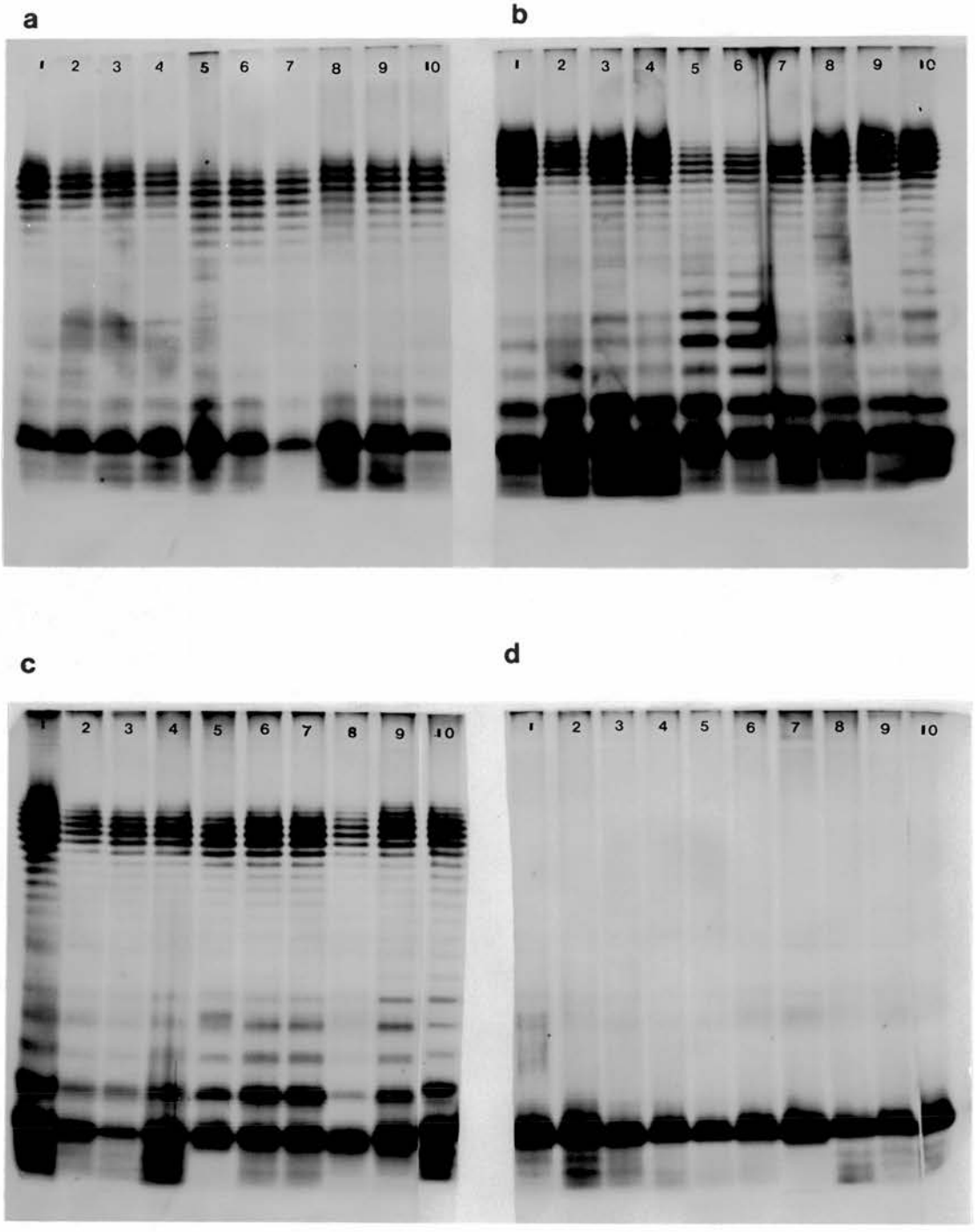


Figure 78. Silver stained LPS profiles of proteinase K whole cell digests of *E. coli* strains O?:K5 (484) (a); O18:K1 (b); O18:K1 (c) and O18:Krf (d) separated by PAGE (14% w/v acrylamide). For each strain, Track 1 represents cells grown in the absence of antibiotic and Tracks 2-4, 5-7 and 8-10 represent cells grown in the presence of 1:2, 1:4 and 1:8 MIC dilutions of ampicillin, chloramphenicol and gentamicin respectively.

NA = no antibiotic, Amp = ampicillin, Chlor = chloramphenicol and Gent = gentamicin.

O18, O-antigen specific MAb 184.2.5.5 and core-specific MAb 27.150.3 (Figure 79), demonstrates the increased expression of low molecular mass LPS material from cells exposed to chloramphenicol (Tracks 5-7), compared to untreated cells. Immunoblot analysis of antibiotic treated and untreated cells of *E. coli* O18:K5 (226) against 27.150.3 (Figure 80), again highlights the greater reactivity against low molecular mass LPS bands of chloramphenicol treated cells.

Figures 81 and 82 represent the silver stained LPS PAGE profiles and their corresponding immunoblots (probed with MAb 27.150.3) of eight *E. coli* strains grown in the absence and presence of sub-MICs of ciprofloxacin. LPS of ciprofloxacin treated cells showed only minor changes against untreated control cells, including a small increase in the O-polysaccharide chain length for some strains (Figure 82., Tracks 2-4). Probing LPS profiles of the four *E. coli* O18 smooth strains with O18-serotype specific MAb 184.2.5.5, again demonstrates an increased O-polysaccharide chain length for three of the four strains, notably at a ciprofloxacin MIC dilution of 1:2 (Figure 83b-d).

4.2 BINDING OF ANTI-LIPOPOLYSACCHARIDE MONOCLONAL ANTIBODIES TO ANTIBIOTIC TREATED BACTERIA IN ELISA

Viable bacteria, grown to early stationary phase in the absence and presence of 1:2, 1:4 and 1:8 MIC dilutions of antibiotics were used to coat microtitre plates. Coated whole cells were probed with four anti-LPS MAbs: O18, O-antigen specific MAb 184.2.5.5; outer-core-specific MAb 43.3.4.8; Rc core-specific MAb 27.150.3 and Re core-specific MAb 43.27.11.2.

ELISA-titres were defined as the dilution of MAb that showed an OD of

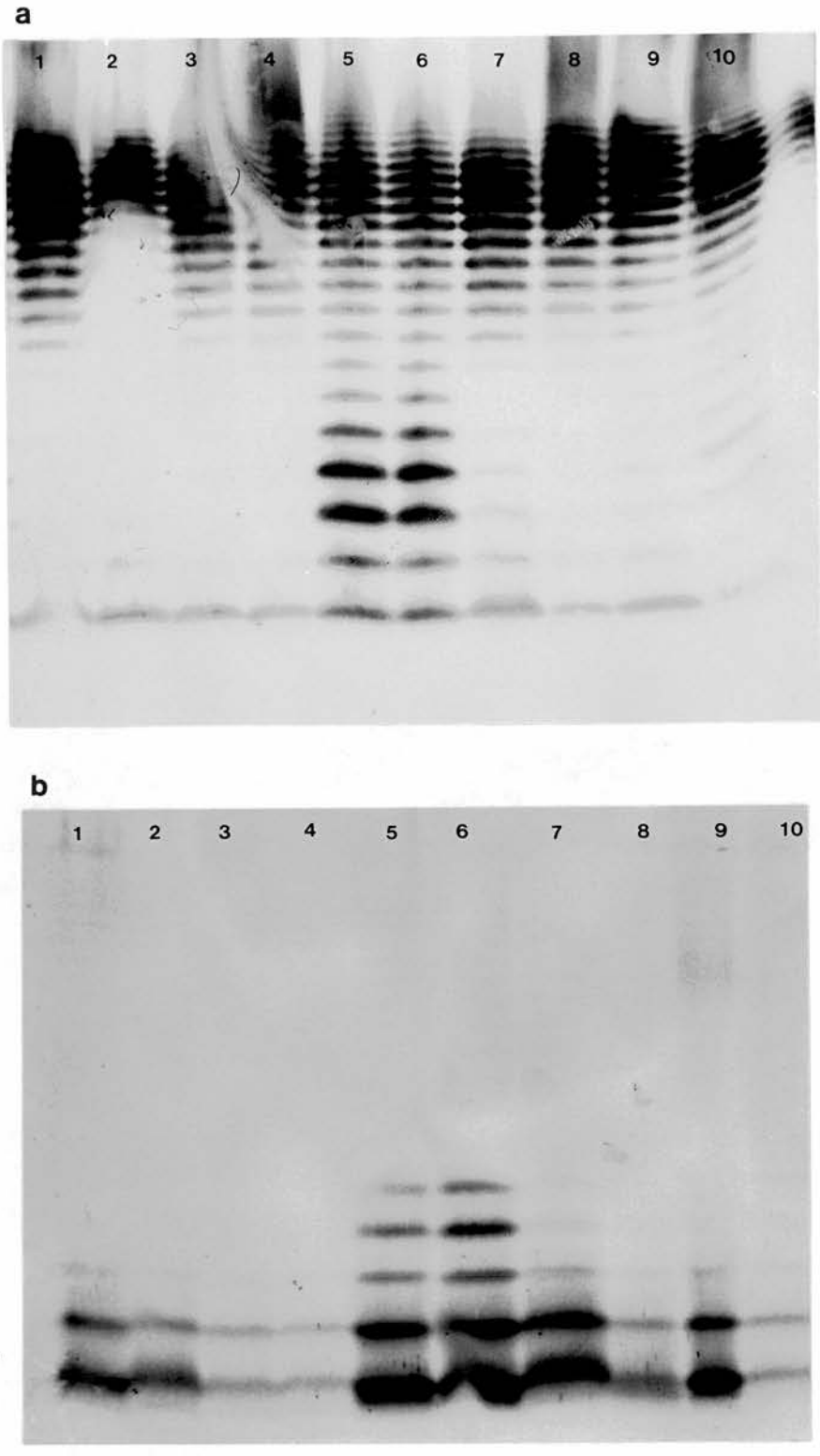


Figure 79. Immunoblots of proteinase K whole cell digests of *E. coli* O18:K1 separated by PAGE (14% w/v acrylamide) followed by transfer to NIC paper and probed with O-antigen specific MAb 184.2.5.5 (a) and core-specific MAb 27.150.3 (b). Track 1 represents cells grown in the absence of antibiotic and Tracks 2-4, 5-7 and 8-10 represent cells grown in the presence of 1:2, 1:4 and 1:8 MIC dilutions of ampicillin, chloramphenicol and gentamicin respectively.

NA = no antibiotic, Amp = ampicillin, Chlor = chloramphenicol and Gent = gentamicin.

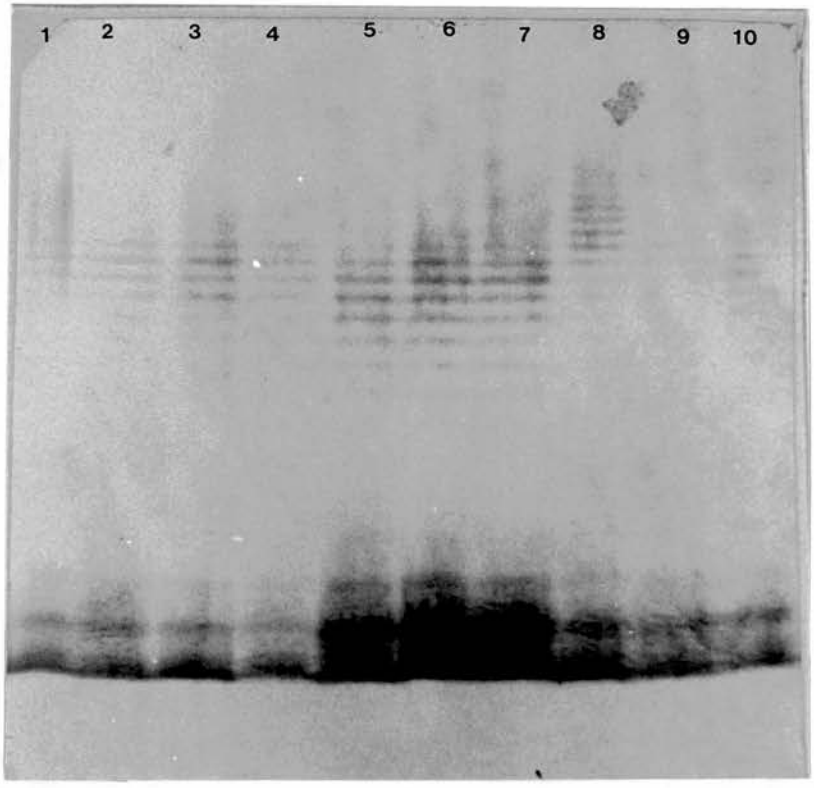


Figure 80. Immunoblot of proteinase K whole cell digests of *E. coli* O?:K5 (484) separated by PAGE (14% w/v acrylamide) followed by transfer to NIC paper and probed with core-specific MAb 27.150.3. Track 1 represents cells grown in the absence of antibiotic and Tracks 2-4, 5-7 and 8-10 represent cells grown in the presence of 1:2, 1:4 and 1:8 MIC dilutions of ampicillin, chloramphenicol and gentamicin respectively.

NA = no antibiotic, Amp = ampicillin, Chlor = chloramphenicol and Gent = gentamicin.

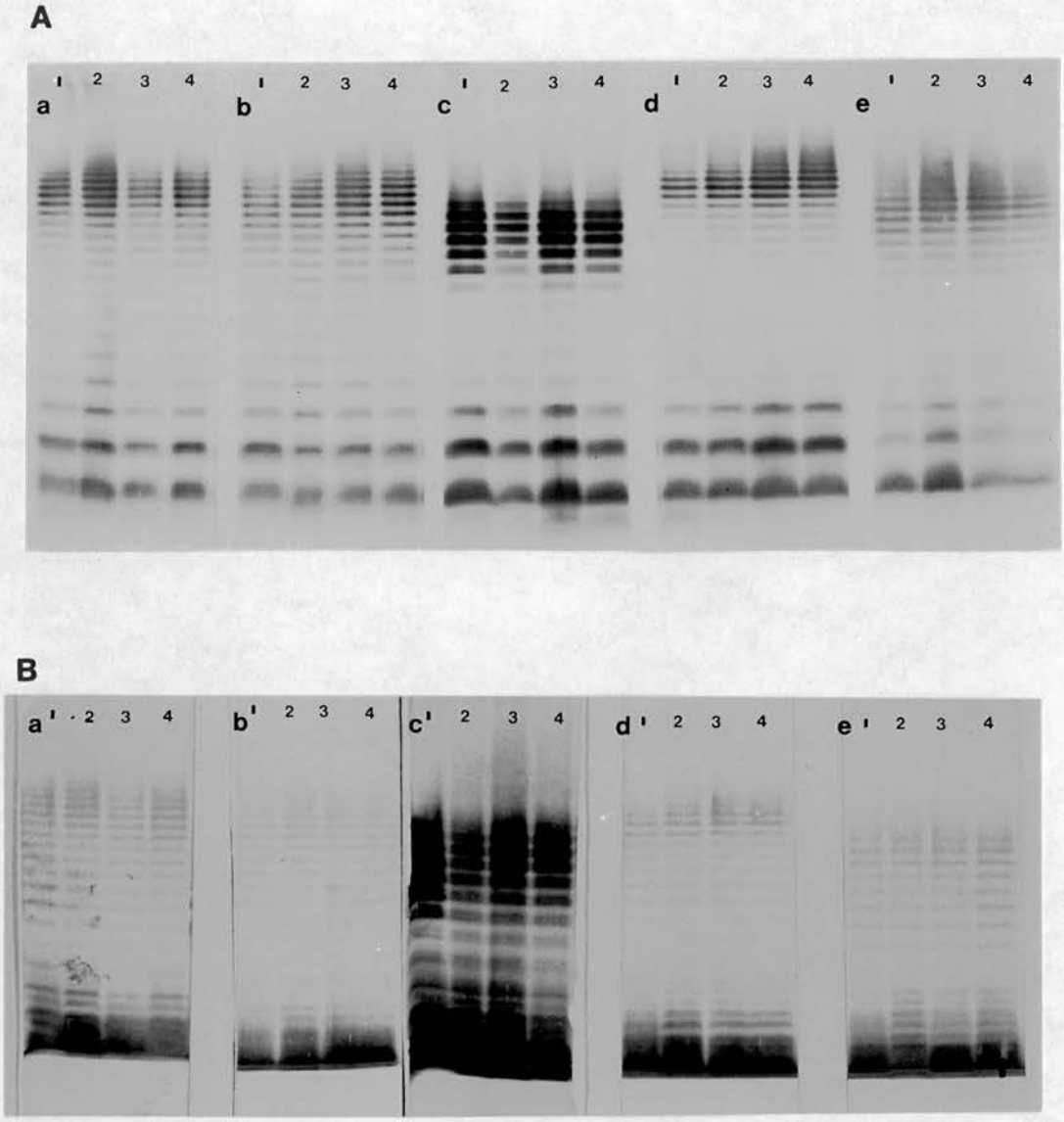


Figure 81. A) Silver stained LPS profiles of proteinase K whole cell digests of *E. coli* strains O18:K5 (226) (a); O18:K5 (281) (b); O1:K1 (316) (c); O6:K? (317) (d) and O?:K5 (484) (e) separated by PAGE (14% w/v acrylamide). B) Immunoblot of the equivalent proteinase K whole cell digests separated by PAGE (14% w/v acrylamide) followed by transfer to NIC paper and probed with core-specific MAb 27.150.3. For each strain, Track 1 represents cells grown in the absence of antibiotic and Tracks 2-4 represent cells grown in the presence of 1:2, 1:4 and 1:8 MIC dilutions of ciprofloxacin.

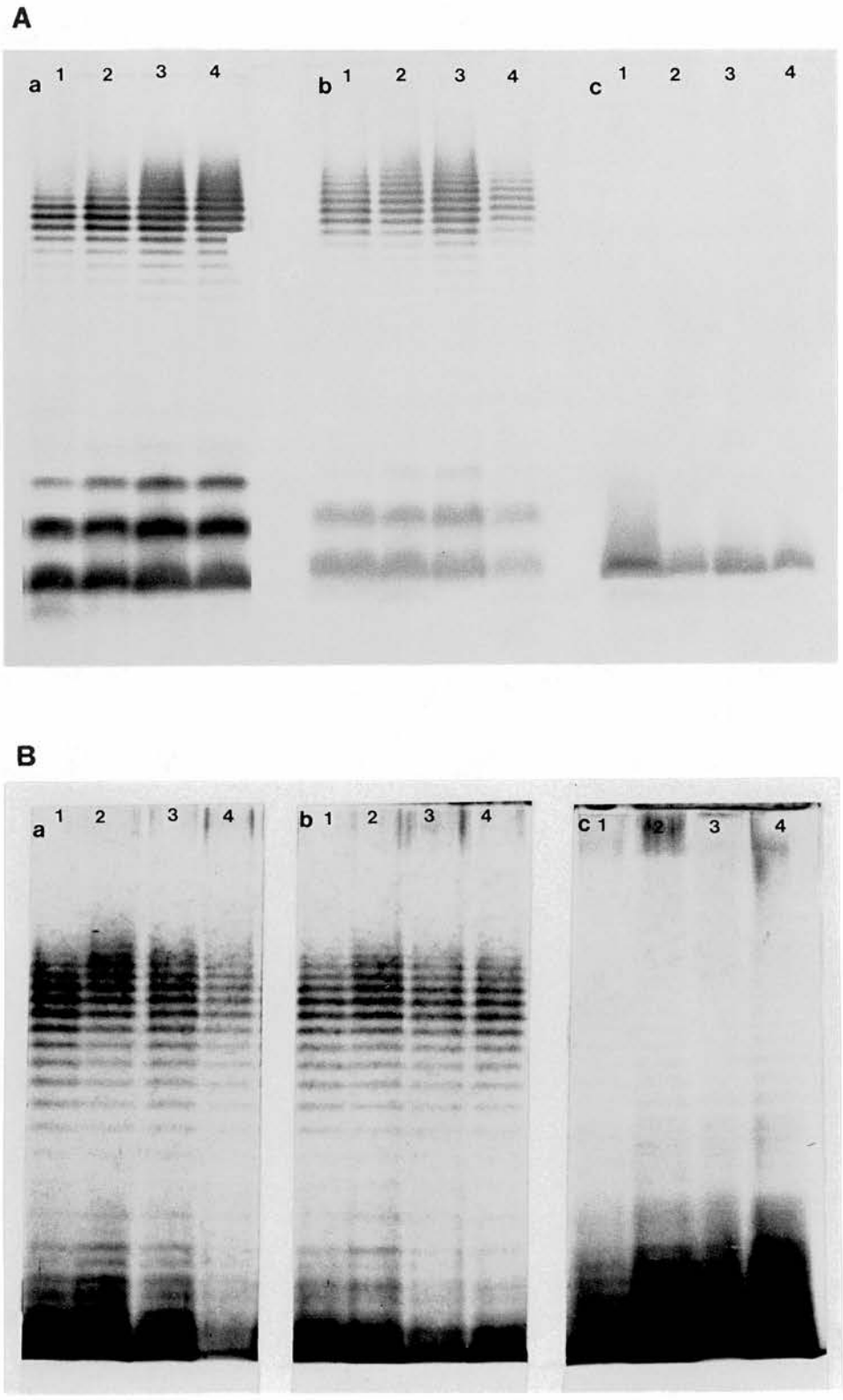


Figure 82. A) Silver stained LPS profiles of proteinase K whole cell digests of *E. coli* strains O18:K1 (a), O18:K1 (b) and O18:Krf (c) separated by PAGE (14% w/v acrylamide). B) Immunoblot of the equivalent proteinase K whole cell digests separated by PAGE (14% w/v acrylamide) followed by transfer to NIC paper and probed with core-specific MAb 27.150.3. For each strain, Track 1 represents cells grown in the absence of antibiotic and Tracks 2-4 represent cells grown in the presence of 1:2, 1:4 and 1:8 MIC dilutions of ciprofloxacin.

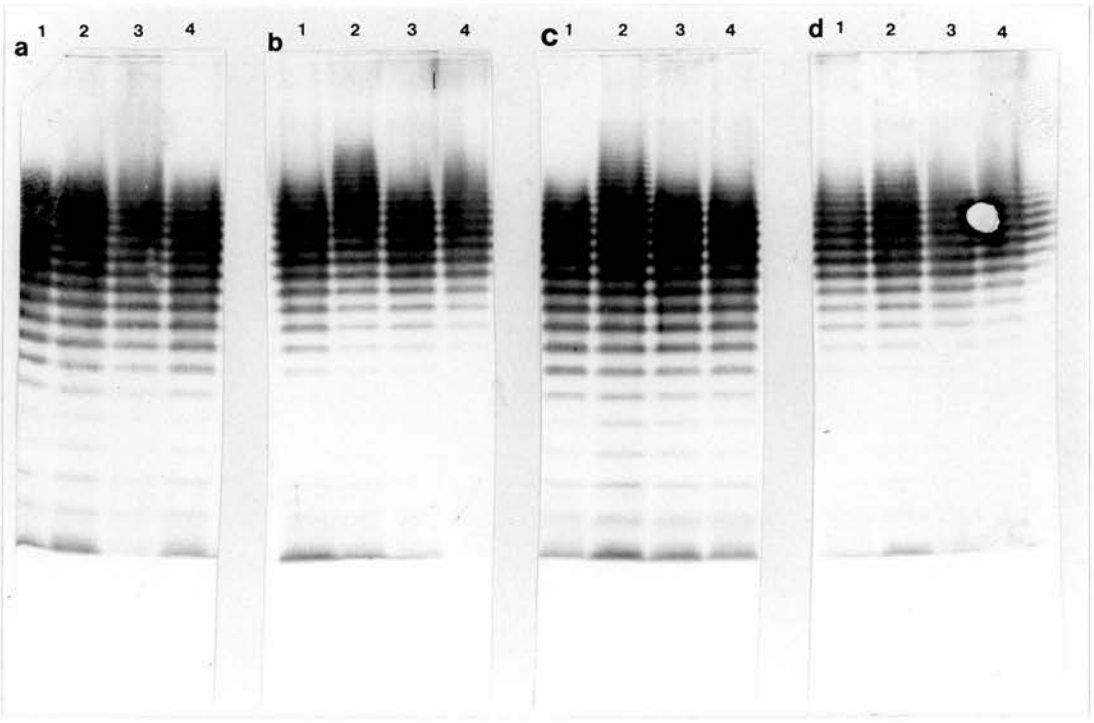


Figure 83. Immunoblot of proteinase K whole cell digests of *E. coli* O18:K5 (226) (a); O18:K5 (281) (b); O18:K1 (c) and O18:K1 (d) separated by PAGE (14% w/v acrylamide) followed by transfer to NIC paper and probed with O-antigen specific MAb 184.2.5.5. For each strain, Track 1 represents cells grown in the absence of antibiotic and Tracks 2-4 represents cells grown in the presence of 1:2, 1:4 and 1:8 MIC dilutions of ciprofloxacin.

0.2 at 590 nm. If an OD of 0.2 was not reached, the reaction was considered negative. Since differences in coating-efficiency may occur between the different antigens, ELISA-titre ratios were compared instead of the measured absolute titres (Overbeek *et al*, 1989). The ratio was defined as the titre obtained with a certain MAb to a strain grown in the presence of antibiotic, divided by the titre obtained with the same MAb to this strain grown in the absence of antibiotic. A ratio of more than one indicates an increase in binding of MAb to the strain grown in the presence of antibiotic. However, growth of bacteria in the presence of antibiotic may increase the coating efficiency of antigens to plates, resulting in ratios of more than one, not necessarily reflecting an increase in MAb binding. Therefore, in terms of enhanced MAb binding, only ratios of more than two were considered significant.

ELISA-titre ratios of anti-LPS MAb against eight *E. coli* strains grown in the absence or presence of antibiotics are presented in Tables 13-16. Overall, treatment of cells with various antibiotics did not alter the binding of O-antigen specific MAb 184.2.5.5. Any significant differences that did occur were in cells treated with ciprofloxacin, illustrated by *E. coli* O18:K1$^-$, 1:2 MIC dilution (Table 16).

The three anti-core MAbs all showed increased reactivity against some or all smooth *E. coli* grown in the presence of antibiotics compared to untreated cells. Those antibiotics inducing the greatest increases included ampicillin, chloramphenicol and ciprofloxacin. Gentamicin was shown to exert the least effect on MAb binding, although significant increases against some strains were observed. Exposure of *E. coli* O6:K? (317) and O18:K1$^-$ to antibiotics had the least effect on MAb

Table 13. ELISA-titre ratios obtained with 4 anti-LPS MAbs to *E. coli* O18:K5 (226) and O18:K5 (281) grown in the presence or absence of sub-MICs of antibiotics. MAbs include O18, O-antigen specific MAb 184.2.5.5 and core-specific MAbs 27.150.3, 43.3.4.8 and 43.27.11.2. Amp = Ampicillin; Chlor = Chloramphenicol; Gent = Gentamicin; Cipro = Ciprofloxacin. Results were calculated from the means of triplicate readings. (| = ≤ 1)

	Antibiotic and MIC dilution											
***E. coli* O18:K5 (226)**	Amp			Chlor			Gent			Cipro		
MAb	1:2	1:4	1:8	1:2	1:4	1:8	1:2	1:4	1:8	1:2	1:4	1:8
184.2.5.5	|	|	|	|	|	|	|	|	|	1	2	1
27.150.3	16	6	7	24	24	3	1	|	|	10	|	|
43.3.4.8	16	16	16	18	9	9	|	|	|	5	1	1
43.27.11.2	229	180	95	53	47	10	1	|	|	69	43	10
***E. coli* O18:K5 (281)**												
MAb	1:2	1:4	1:8	1:2	1:4	1:8	1:2	1:4	1:8	1:2	1:4	1:8
184.2.5.5	1	1	|	|	|	|	|	|	|	1	1	1
27.150.3	14	14	10	20	20	16	10	6	|	12	9	9
43.3.4.8	2	2	1	6	3	3	4	6	|	4	|	|
43.27.11.2	18	18	12	4	2	1	5	5	1	16	16	4

Table 14. ELISA-titre ratios obtained with 4 anti-LPS MAbs to *E. coli* O1:K1 (316) and O6:K? (317) grown in the presence and absence of sub-MICs of antibiotics. MAbs include O18, O-antigen specific MAb 184.2.5.5 and core-specific MAbs 27.150.3, 43.3.4.8 and 43.27.11.2. NC = Not calculated (OD<0.2); Amp = Ampicillin; Chlor = Chloramphenicol; Gent = Gentamicin; Cipro = Ciprofloxacin. Results were calculated from the means of triplicate readings. (1 = ≤1)

	Antibiotic and MIC dilution											
***E. coli* O1:K1 (316)**												
	Amp			Chlor			Gent			Cipro		
MAb	1:2	1:4	1:8	1:2	1:4	1:8	1:2	1:4	1:8	1:2	1:4	1:8
184.2.5.5	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC
27.150.3	1	4	1	86	93	8	6	1	1	20	16	17
43.3.4.8	16	16	4	35	30	29	5	4	4	16	16	14
43.27.11.2	7	7	4	2	2	1	1	1	1	9	9	4
***E. coli* O6:K? (317)**												
	Amp			Chlor			Gent			Cipro		
MAb	1:2	1:4	1:8	1:2	1:4	1:8	1:2	1:4	1:8	1:2	1:4	1:8
184.2.5.5	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC
27.150.3	31	1	1	6	1	1	1	1	1	1	1	1
43.3.4.8	15	18	12	4	4	3	1	1	1	4	1	1
43.27.11.2	5	5	5	1	2	1	1	1	1	3	3	1

Table 15. ELISA-titre ratios obtained with 4 anti-LPS MAbs to *E. coli* O?:K5 (484) and O18:K1 grown in the presence or absence of sub-MICs and antibiotics. MAbs include O18, O-antigen specific MAb 184.2.5.5 and core-specific MAbs 27.150.3, 43.3.4.8 and 43.27.11.2. NC = Not calculated (OD<0.2); Amp = Ampicillin; Chlor = Chloramphenicol; Gent = Gentamicin; Cipro = Ciprofloxacin. Results were calculated from the means of triplicate readings. (I = ≤1)

	Antibiotic and MIC dilution											
***E. coli* O?:K5 (484)**	Amp			Chlor			Gent			Cipro		
MAb	1:2	1:4	1:8	1:2	1:4	1:8	1:2	1:4	1:8	1:2	1:4	1:8
184.2.5.5	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC
27.150.3	16	64	16	1	1	1	62	62	55	14	10	10
43.3.4.8	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC
43.27.11.2	6	1	1	8	5	5	1	I	I	I	I	I
***E. coli* O18:K1**	Amp			Chlor			Gent			Cipro		
MAb	1:2	1:4	1:8	1:2	1:4	1:8	1:2	1:4	1:8	1:2	1:4	1:8
184.2.5.5	1	1	1	I	I	I	1	I	1	1	I	I
27.150.3	16	16	4	8	8	8	1	1	1	13	8	10
43.3.4.8	4	1	1	1	1	1	4	1	1	3	3	I
43.27.11.2	3	2	2	6	8	4	3	I	I	6	7	6

Table 16. ELISA-titre ratios obtained with 4 anti-LPS MAbs to *E. coli* O18:K1⁻ and O18:Krf grown in the presence or absence of sub-MICs of antibiotics. MAbs include O18, O-antigen specific MAb 184.2.5.5 and core-specific MAbs 27.150.3, 43.3.4.8 and 43.27.11.2. Amp = Ampicillin; Chlor = Chloramphenicol; Gent = Gentamicin; Cipro = Ciprofloxacin. Results were calculated from the means of triplicate readings. (1 = ≤1)

	Antibiotic and MIC dilution											
***E. coli* O18:K1⁻**	Amp			Chlor			Gent			Cipro		
MAb	1:2	1:4	1:8	1:2	1:4	1:8	1:2	1:4	1:8	1:2	1:4	1:8
184.2.5.5	1	1	1	1	1	1	1	1	1	3	1	1
27.150.3	16	16	4	6	6	2	1	1	1	7	8	5
43.3.4.8	1	1	1	1	1	1	1	1	1	1	1	1
43.27.11.2	4	3	3	13	16	16	1	1	1	4	4	1
***E. coli* O18:Krf**	Amp			Chlor			Gent			Cipro		
MAb	1:2	1:4	1:8	1:2	1:4	1:8	1:2	1:4	1:8	1:2	1:4	1:8
184.2.5.5	1	1	1	1	1	1	1	1	1	2	1	1
27.150.3	4	1	1	1	1	1	1	1	1	1	1	1
43.3.4.8	1	1	1	1	1	1	1	1	1	1	1	1
43.27.11.2	1	1	1	1	1	1	1	1	1	1	1	1

binding compared to other untreated smooth strains. Growth of the rough mutant, *E. coli* O18:Krf in the presence of antibiotics showed only occasional increases in MAb reactivity.

4.3 FLOW CYTOMETRIC ANALYSIS OF THE BINDING OF ANTI-LIPOPOLYSACCHARIDE MONOCLONAL ANTIBODIES TO ANTIBIOTIC TREATED BACTERIA

To investigate changes in binding activities of anti-LPS MAbs to whole cells exposed to sub-MICs of antibiotics further, flow cytometric analysis was performed. Figures 84-86 show the flow cytometric profiles of MAbs to selected whole cells grown in the absence or presence of one half of the MIC of various antibiotics.

The fluorescence patterns produced by O18, O-antigen specific MAb 184.2.5.5 against treated and untreated cells varied considerably for each *E. coli* strain. Exposure of *E. coli* O18:K5 (281) to the antibiotics gentamicin and ciprofloxacin in particular resulted in cells exhibiting greater fluorescence intensity (Figure 84a). However, both antibiotic treated and untreated samples showed similar percentage cells exhibiting positive fluorescence. Significant changes in positive fluorescence levels for *E. coli* O18:K1 probed with 184.2.5.5 were observed for cells exposed to chloramphenicol (45%) and ciprofloxacin (87%) compared to untreated cells (70%). The presence of small amounts of O-antigen on the rough mutant (O18:Krf) reflects low binding levels of 184.2.5.5 (Figure 86a). Those cells exposed to ampicillin and chloramphenicol demonstrated highest positive fluorescence values.

The core-specific MAbs 27.150.3 and 43.3.4.8 exhibited similar levels of binding to cells of the rough mutant, *E. coli* O18:Krf, grown in the

Figure 84. Flow cytometry. Green fluorescence intensity histograms of three anti-LPS MAbs against whole cells of E. coli 018:K5 (281) grown in the absence or presence of one half of the MIC of various antibiotics. MAbs include 018, O-antigen specific MAb 184.2.5.5 a) and two core-specific MAbs 27.150.3 b) and 43.3.4.8 c). Percentage values represent bacteria exhibiting positive fluorescence above background levels. NA = no antibiotic; Amp = ampicillin; Chlor = chloramphenicol; Gent = gentamicin; Cipro = ciprofloxacin.

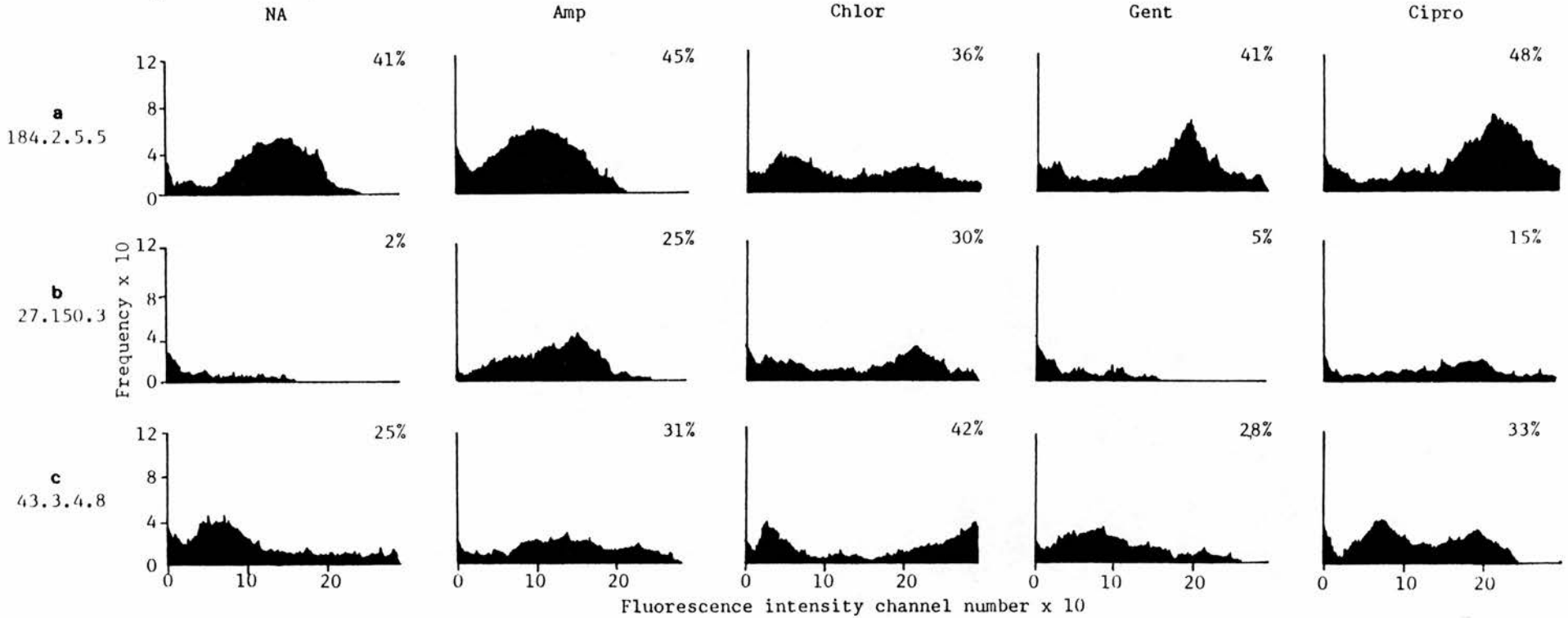


Figure 85. Flow cytometry. Green fluorescence intensity histograms of three anti-LPS MAbs against whole cells of E. coli 018:K1 grown in the absence or presence of one half of the MIC of various antibiotics. MAbs include 018, O-antigen specific MAb 184.2.5.5 a) and two core-specific MAb 27.150.3 b) and 43.3.4.8 c). Percentage values represent bacteria exhibiting positive fluorescence above background levels. NA = no antibiotic; Amp = ampicillin; Chlor = chloramphenicol; Gent = gentamicin; Cipro = ciprofloxacin.

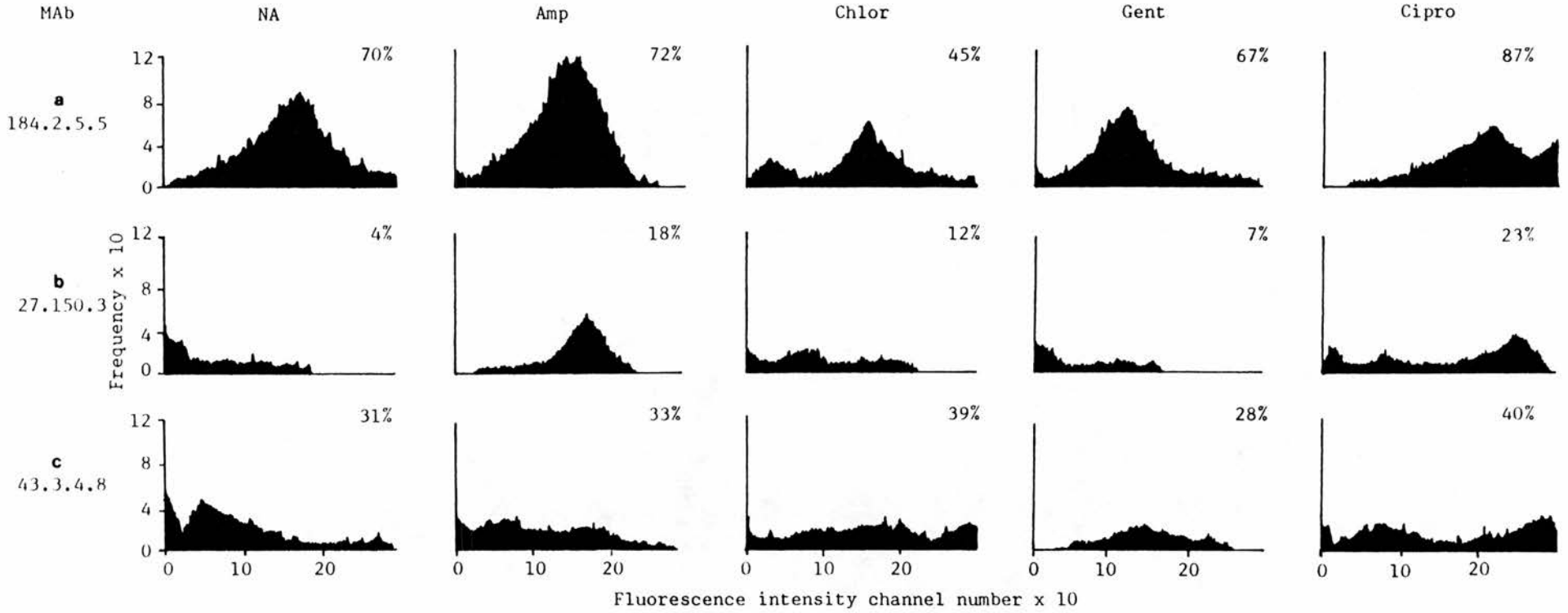
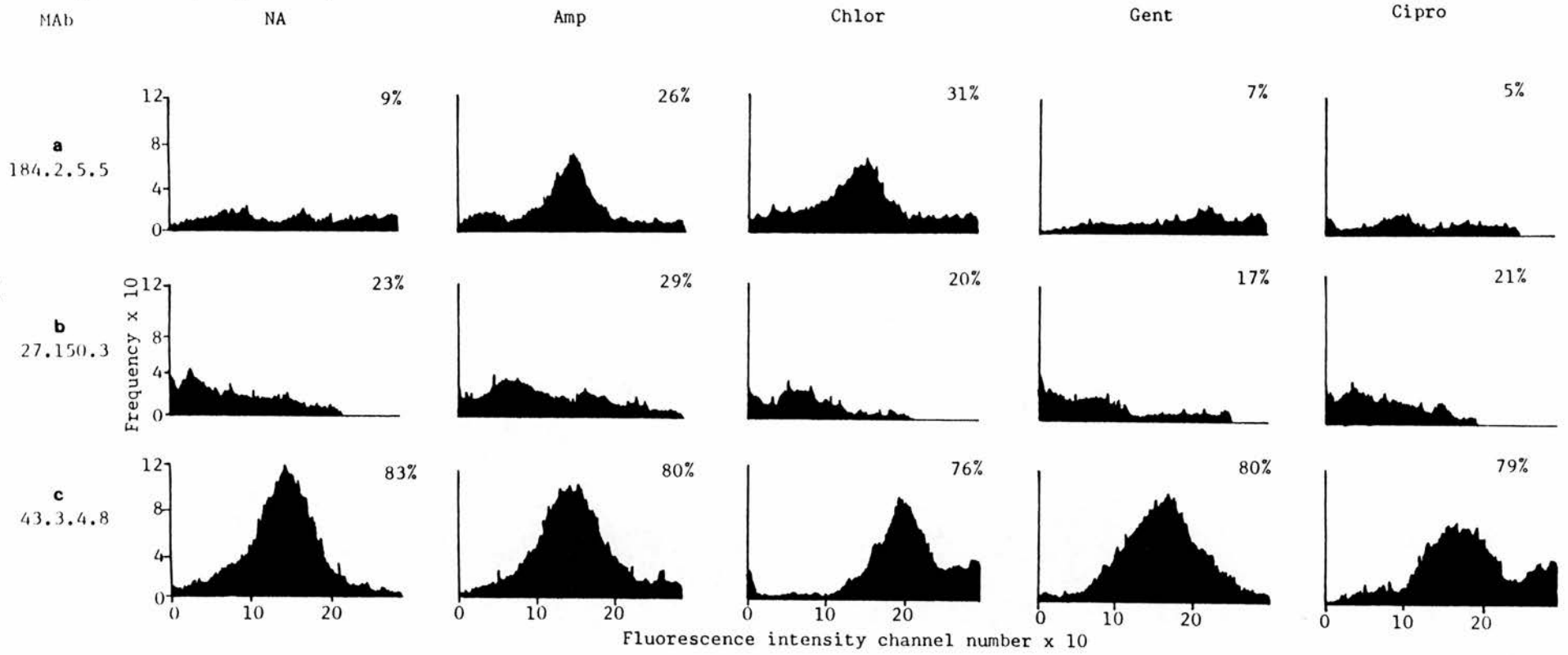


Figure 86. Flow cytometry. Green fluorescence intensity histograms of three anti-LPS MAbs against whole cells of E. coli O18:Krf grown in the absence or presence of one half of the MIC of various antibiotics. MAbs include O18, O-antigen specific MAb 184.2.5.5 a) and two core-specific MAbs 27.150.3 b) and 43.3.4.8 c). Percentage values represent bacteria exhibiting positive fluorescence above background levels. NA = no antibiotic; Amp = ampicillin; Chlor = chloramphenicol; Gent = gentamicin; Cipro = ciprofloxacin.



presence or absence of antibiotics (Figure 86b & c). Mab 27.150.3 showed weak binding levels (< 5%) to smooth *E. coli* O18:K1 (Figure 85b) and O18:K5 (281) (Figure 84b) not exposed to antibiotics. However, growth of these strains in the presence of sub-MIC levels of ampicillin, chloramphenicol and ciprofloxacin all resulted in significant increases in positive fluorescence levels when probed with 27.150.3. Those samples exhibiting an increased percentage of cells with positive fluorescence also demonstrated greater fluorescence intensity levels as shown by differences in the flow cytometric profiles of antibiotic treated and untreated cells. Small increases in positive fluorescence values were observed for most smooth *E. coli*, exposed to the different antibiotics and probed with MAb 43.3.4.8. The greatest increase was shown for *E. coli* O18:K5 (281), grown in the presence of chloramphenicol (42%) against cells grown in the absence of antibiotics (25%) (Figure 84c). Gentamicin induced only minor increases in binding of MAbs 27.150.3 and 43.3.4.8 to smooth strains, against untreated control cells.

A number of antibiotic treated samples also produced biphasic fluorescence patterns as shown in Figures 84-86 indicating the presence of subpopulations of cells.

4.4 OUTER MEMBRANE PROTEIN ANALYSIS OF ANTIBIOTIC TREATED *E. COLI*

SDS-PAGE was used to analyse the effects of one half of the MIC of various antibiotics on the expression of outer membrane proteins of six *E. coli* strains (Figures 87 and 88). The outer membrane proteins of cells grown in the absence or presence of subinhibitory concentrations of the antibiotics ampicillin, chloramphenicol and gentamicin are shown in Figure 87. The most prominent changes in proteins involved the

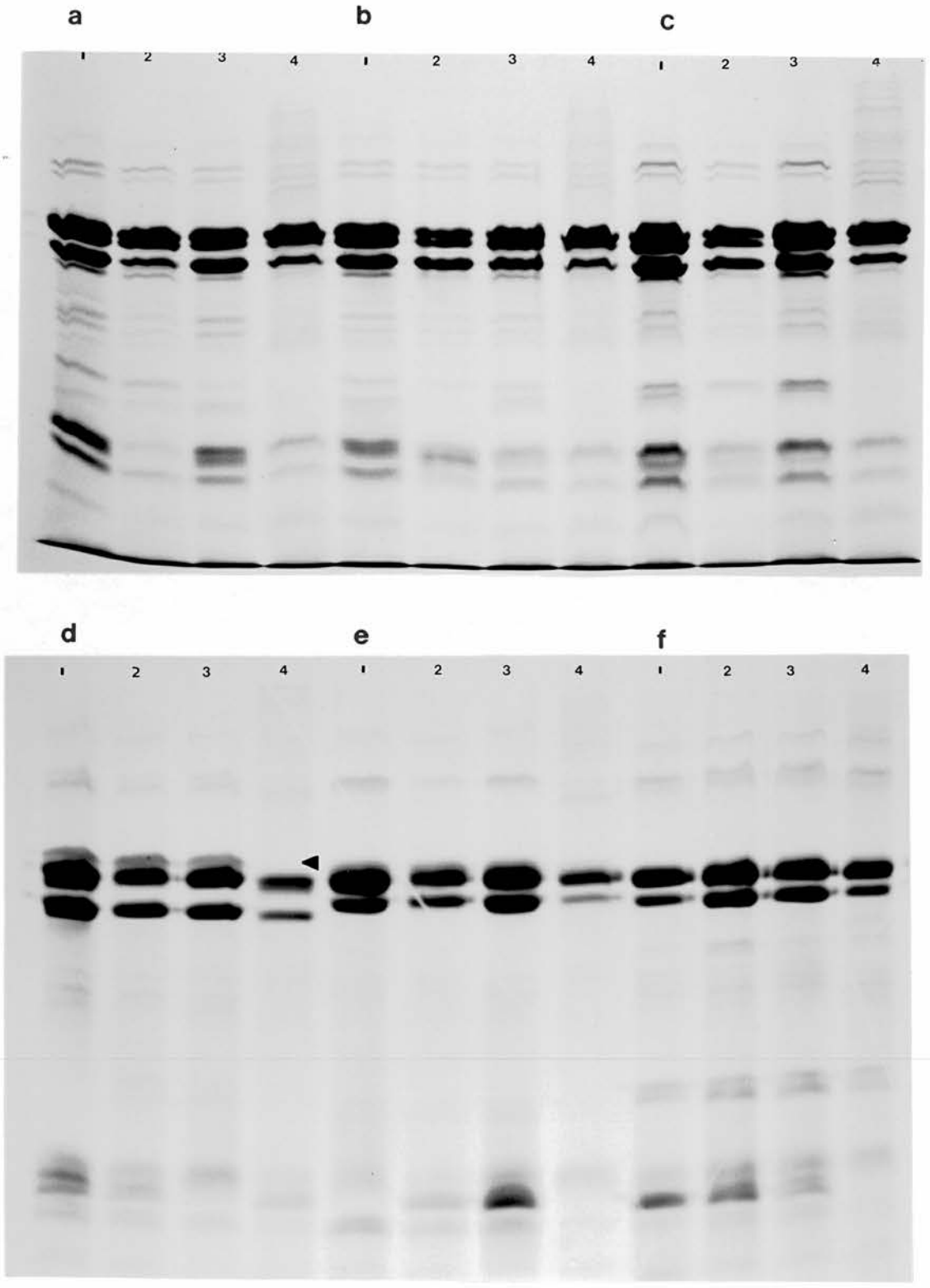


Figure 87. SDS-PAGE of Sarkosyl outer membrane proteins of *E. coli* strains O18:K1 (a); O18:K1$^-$ (b); O18:Krf (c); O18:K5 (281) (d); O6:K? (317) (e) and O?:K5 (484) (f) separated on acrylamide 10% slab gels stained with Coomassie blue. Track 1 represents cells grown in the absence of antibiotic and Tacks 2-4, cells grown in the presence of one half of the MIC of ampicillin, chloramphenicol and gentamicin respectively. The arrowhead indicates the absence of a protein band from *E. coli* O18:K5 exposed to gentamicin.

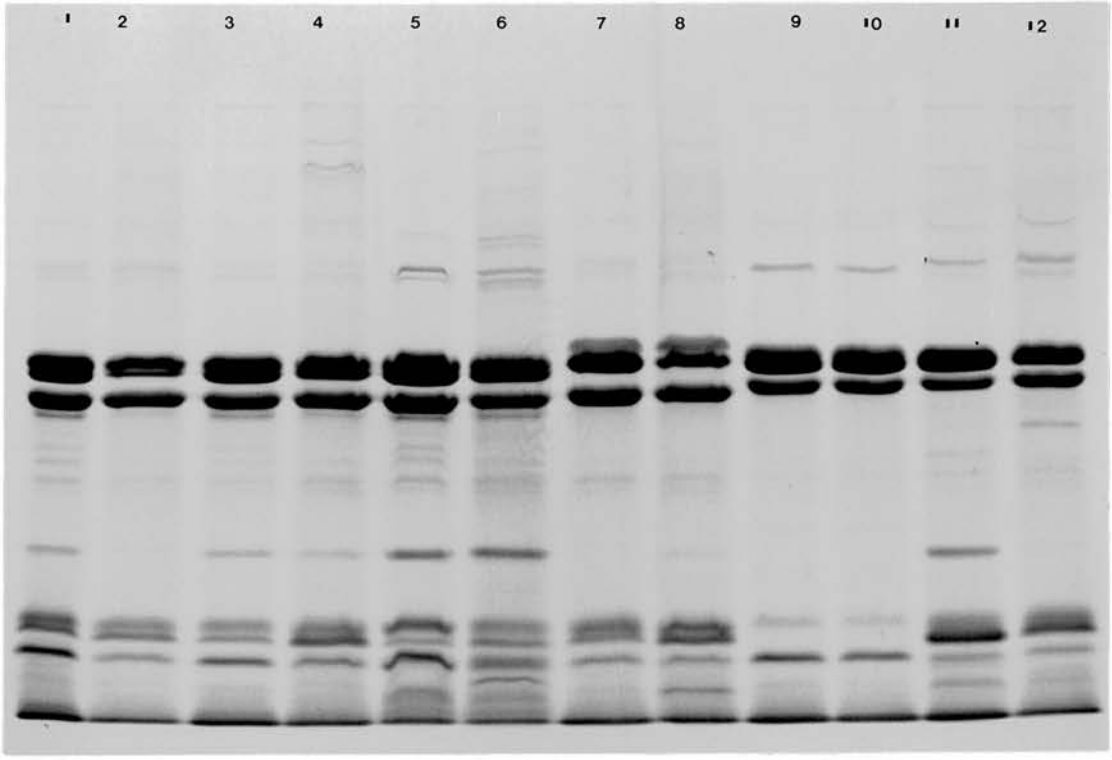


Figure 88. SDS-PAGE of Sarkosyl outer membrane proteins of six *E. coli* strains separated on acrylamide 10% slab gels stained with Coomassie blue. Cells were grown in the absence of antibiotic or presence of one half of the MIC of ciprofloxacin. Tracks 1 and 2 represent O18:K1; Tracks 3 and 4 O18:K1 ; Tracks 5 and 6 O18:Krf; Tracks 7 and 8 O18:K5 (281); Tracks 9 and 10 O6:K? (317) and Tracks 11 and 12 O?:K5 (484). The first track of each strain represents cells grown in the absence of antibiotic.

appearance of several minor high molecular mass proteins (> 45 kDa) in most *E. coli* strains exposed to gentamicin (Track 4), which were absent from untreated, control cells. The ampicillin resistant strain of *E. coli* O18:K5 (281) expressed a protein of approximately 40 kDa for all samples except those cells grown in the presence of gentamicin (absent protein band indicated by arrowhead) (Figure 87d).

The outer membrane proteins of *E. coli* strains grown in the absence and presence of ciprofloxacin are shown in Figure 88. *E. coli* O18:K1$^-$ (Track 4), O18:Krf (Track 6) and O18:K5 (281) (Track 8) expressed several additional minor high molecular mass proteins (> 45 kDa) when exposed to ciprofloxacin, which were absent from untreated cells. No changes involving the major outer membrane proteins were evident.

CHAPTER 5

ANALYSIS OF THE WHOLE CELL ELISA TECHNIQUE

The whole cell ELISA technique, using bacteria attached to the solid-phase matrix of microtitre plates was used to monitor the binding of anti-LPS MAbs in studies previously described. Failure of some of the anti-core LPS MAbs to react against whole cells in flow cytometry, suggested possible differences in antigenic presentation of these suspended cells, compared to those used in the whole cell ELISA technique. The aim of this work, was to establish if binding manipulations of the assay altered the accessibility of LPS epitopes on nutrient broth grown coated whole cells, compared to their native whole cells.

5.1 BINDING ACTIVITIES OF ANTI-LIPOPOLYSACCHARIDE MONOCLONAL ANTIBODIES TO WHOLE CELLS, HEAT-KILLED CELLS AND SONICATED CELLS IN ELISA

Initial investigations were designed to establish if different cell preparations affected the accessibility of LPS epitopes. ELISA activities of two anti-LPS MAbs against whole cells, heat-killed cells (15 min at 100°C) and sonicated cells (six, 1 min bursts with 30 s intervals) of *E. coli* O18:K1 and O18:Krf were studied. Cell samples were prepared from the same original cell suspension, diluted to a density of $2x10^7$ cells ml^{-1}. The greatest reactivity of O-antigen specific MAb 184.2.5.5 was against sonicated cells, followed by heat-killed cells of each *E. coli* strain (Figure 89). The lowest reactivity was against live whole cells. Core-specific MAb 43.27.11.2 also demonstrated greatest reactivity against sonicated cell preparations of

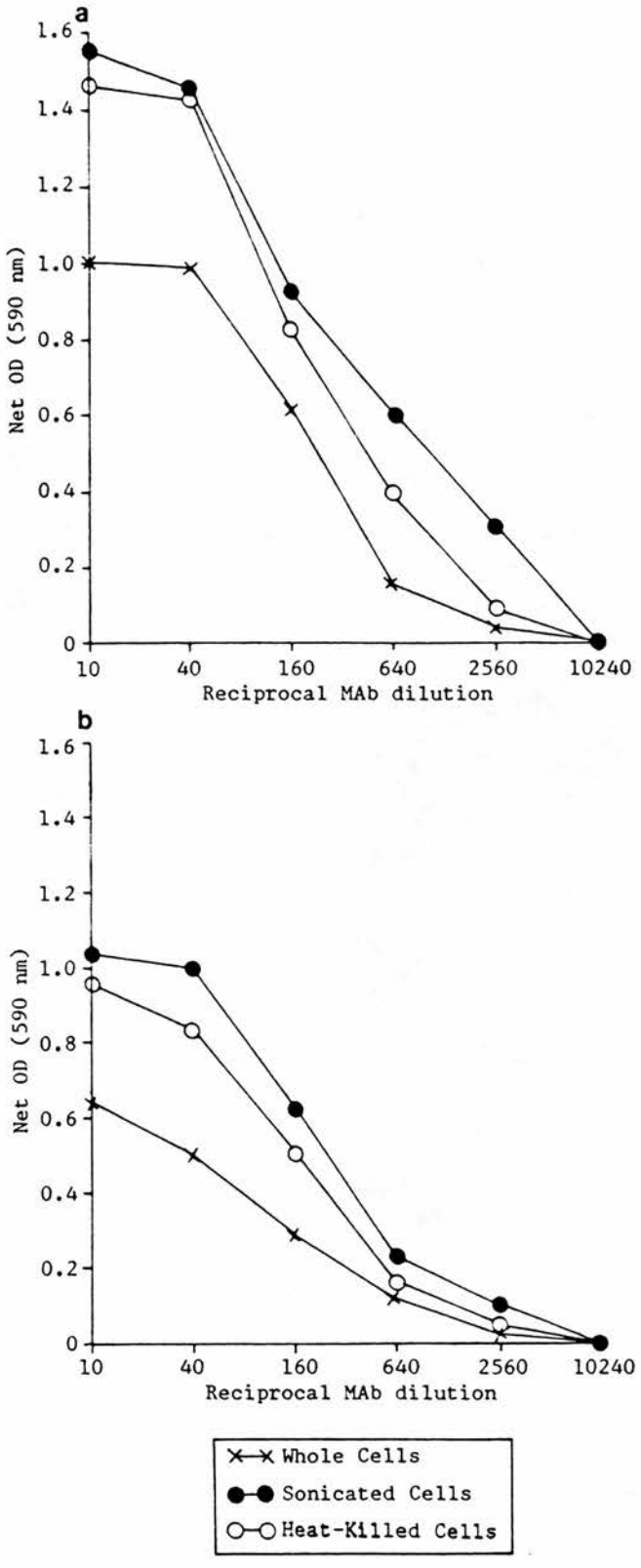


Figure 89. ELISA activity of O18, O-antigen specific MAb, 184.2.5.5 against whole cells, heat-killed cells and sonicated cells of *E. coli* O18:K1 (a) and *E. coli* O18:Krf (b). Results represent the means of triplicate readings.

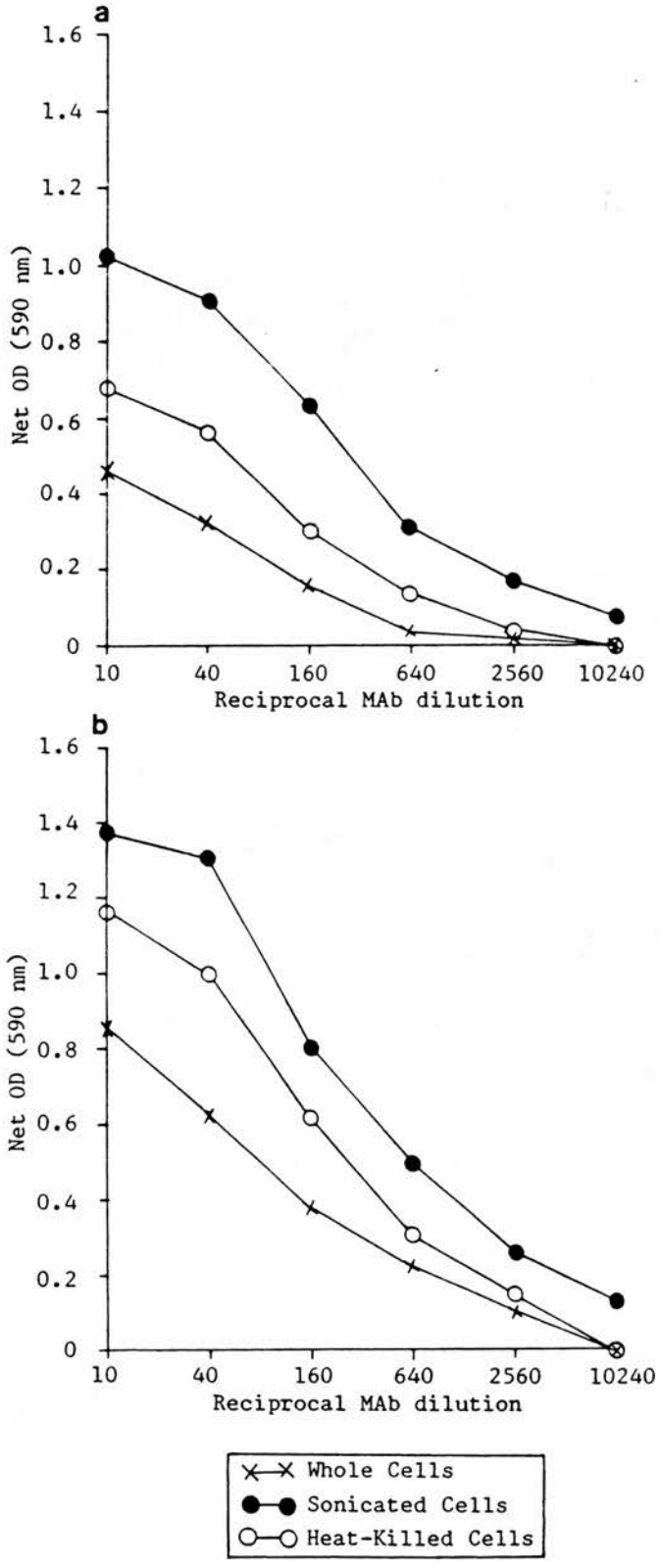


Figure 90. ELISA activity of core-specific MAb, 43.27.11.2 against whole cells, heat-killed cells and sonicated cells of *E. coli* O18:K1 (a) and *E. coli* O18:Krf (b). Results represent the means of triplicate readings.

E. coli O18:K1 and O18:Krf, whilst the lowest reactivity was against their respective whole cell preparations (Figure 90).

5.2 ELISA INHIBITION STUDIES

The ability of whole cells in suspension to inhibit the ELISA activity of anti-LPS MAbs against homologous whole cells coated to microtitre plates at a concentration of $2x10^7$ cells ml^{-1} was also investigated. Four-fold dilutions of MAbs (184.2.5.5 and 43.27.11.2) were pre-incubated with $1x10^9$ cells ml^{-1} at 37°C for 30 min before adding to ELISA. Inhibition of each MAb was compared to uninhibited MAb reactivity. The binding activity of O-antigen specific MAb 184.2.5.5 was completely inhibited by *E. coli* O18:K1 at each MAb dilution (Figure 91a). The same MAb retained some, although reduced activity, after inhibition by the rough mutant O18:Krf at the higher MAb concentrations (Figure 91c). *E. coli* O18:K1 demonstrated limited inhibitory activity against core-specific MAb 43.27.11.2 (Figure 91b), whilst *E. coli* O18:Krf showed significant inhibitory activity against the same MAb (Figure 91d).

Inhibition of ELISA was also performed using a fixed dilution of MAb against ten-fold dilutions of whole cell inhibitor from $1x10^9$ cells ml^{-1}. All inhibitor dilutions were mixed with an equal volume of MAb diluted in dilution buffer (184.2.5.5 1:40, 43.27.11.2 1:10), and preincubated at 37°C for 30 min before adding to ELISA. The inhibitory effect of *E. coli* O18:K1 on the binding of O-antigen specific MAb 184.2.5.5 was shown to increase progressively with an increasing inhibitor concentration (Figure 92a). Residual ELISA activity was completely inhibited at a concentration of $1x10^8$ cells ml^{-1}. The rough mutant *E. coli* O18:Krf also showed an inhibitory effect against MAb 184.2.5.5, although a

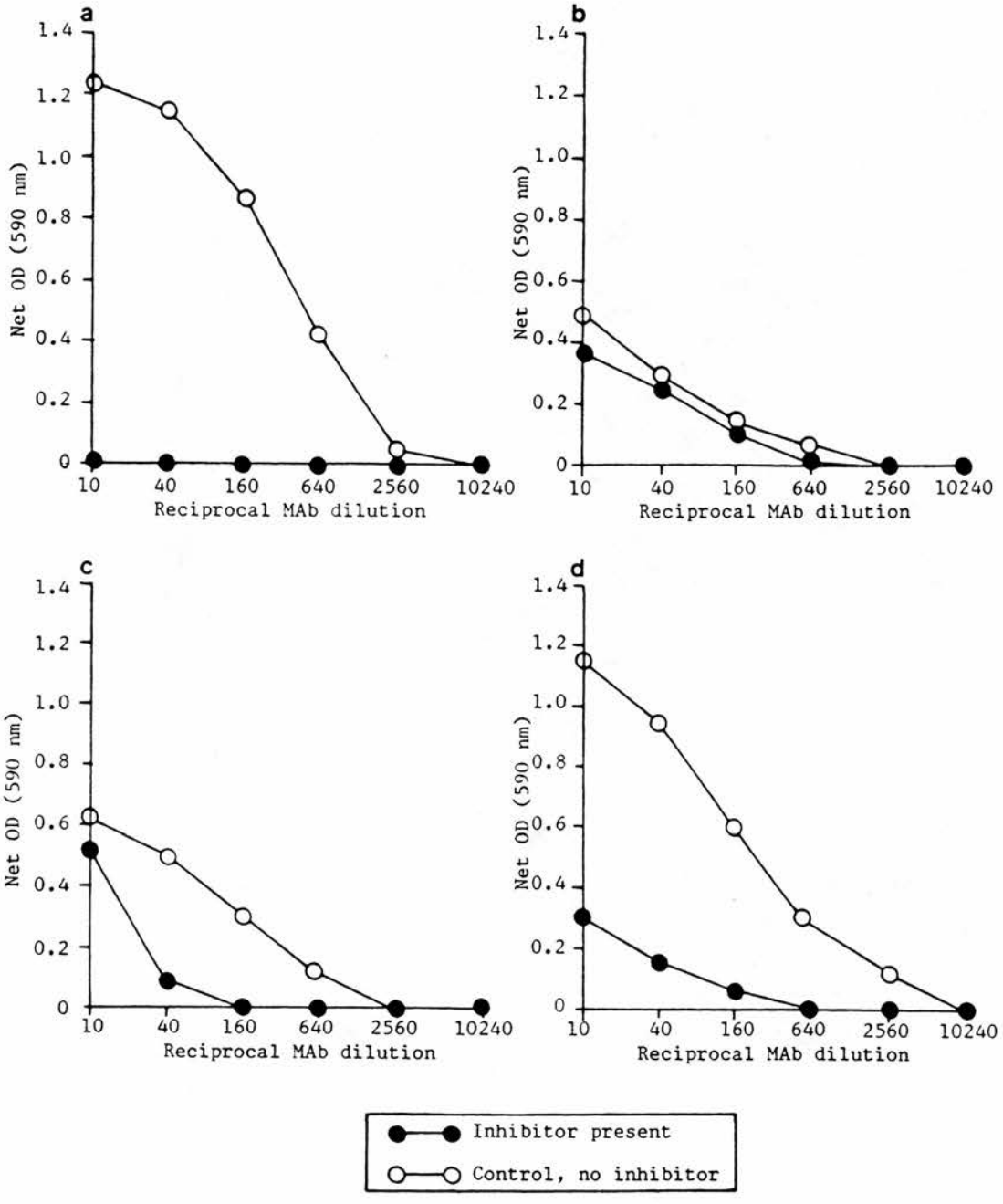


Figure 91. Inhibition of ELISA reactivity of four-fold dilutions of O18, O-antigen specific MAb 184.2.5.5 (a & c) and core-specific MAb 43.27.11.2 (b & d), against the whole cells of *E. coli* O18:K1 (a & b) and its rough mutant O18:Krf (c & d) with homologous whole cells ($1x10^9$ cells ml^{-1}). Controls represent the binding activity of MAbs with no inhibitor present.

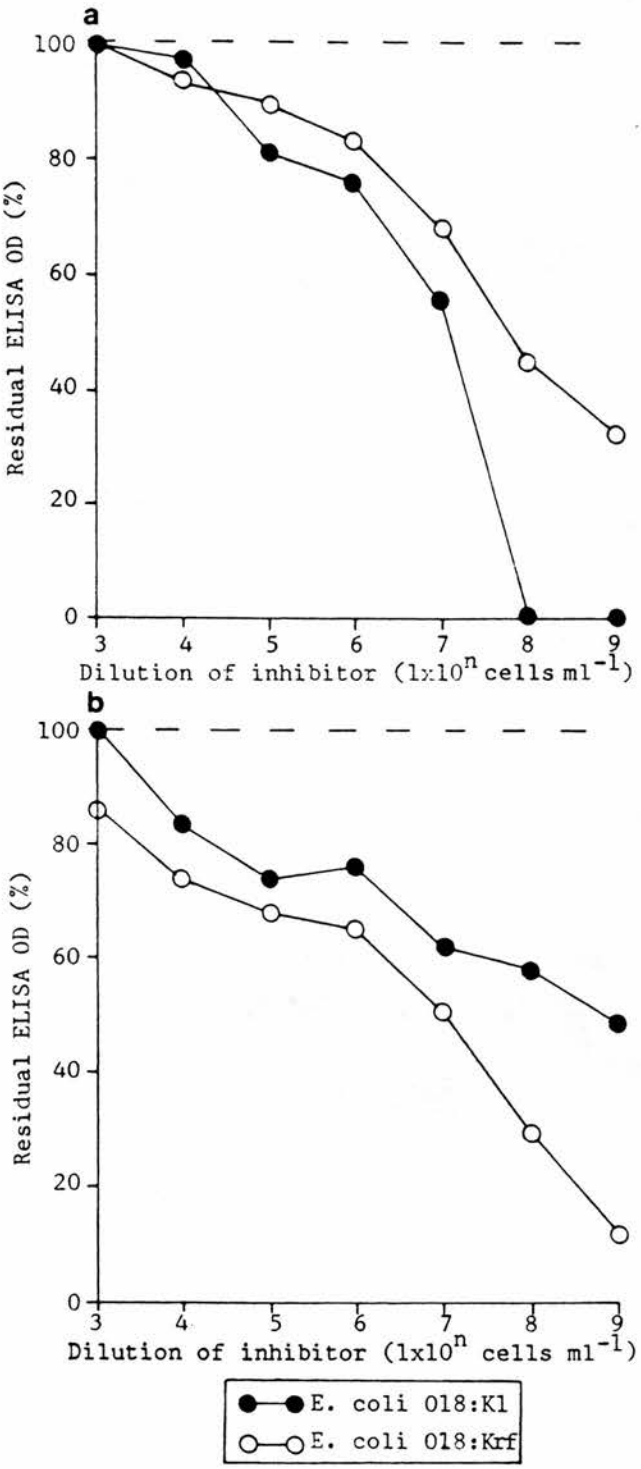


Figure 92. Inhibition of ELISA reactivity of O-specific MAb 184.2.5.5 (1:40) (a) and core-specific MAb 43.27.11.2 (1:10) (b) against whole cells of *E. coli* 018:K1 and its rough mutant 018:Krf with homologous whole cells. Inhibitors were diluted in a ten-fold series from $1x10^9$ ml^{-1} to $1x10^3$ cells ml^{-1}. Residual ELISA reactivity in the presence of inhibitor is expressed as a percentage of reactivity with no inhibitor (the 100% value, represented by the dotted line). Results represent the means of triplicate readings.

residual ELISA activity of 32% was retained at $1x10^9$ cells ml^{-1} inhibitor concentration. The corresponding inhibition of core-specific MAb 43.27.11.2 is shown in Figure 92b). Both *E. coli* O18:K1 and O18:Krf were shown to inhibit MAb binding, although residual ELISA activities of 50% and 12% respectively, were retained at inhibitor concentrations of $1x10^9$ cell ml^{-1}.

5.3 SUSPENSION ELISA

To examine the possible effects of coating cells onto microtitre plates further, the ELISA reactivity of anti-LPS MAbs against whole cells in suspension was compared to coated whole cells. The binding activities of O-antigen specific MAb 184.2.5.5 and core specific MAb 43.27.11.2, against coated or suspended whole cells of *E. coli* O18:K1 and its rough mutant O18:Krf are shown in Figure 93. MAb 184.2.5.5 showed similar reactivity against coated or suspended whole cells of both O18:K1 and O18:Krf (Figure 93a & c). Significant reductions in the binding activity of MAb 43.27.11.2 were shown against suspended whole cells of O18:K1 compared to those cells coated onto microplates (Figure 93b). Suspended cells of O18:Krf showed only minor reductions in binding activity of 43.27.11.2 compared to coated cells (Figure 93d).

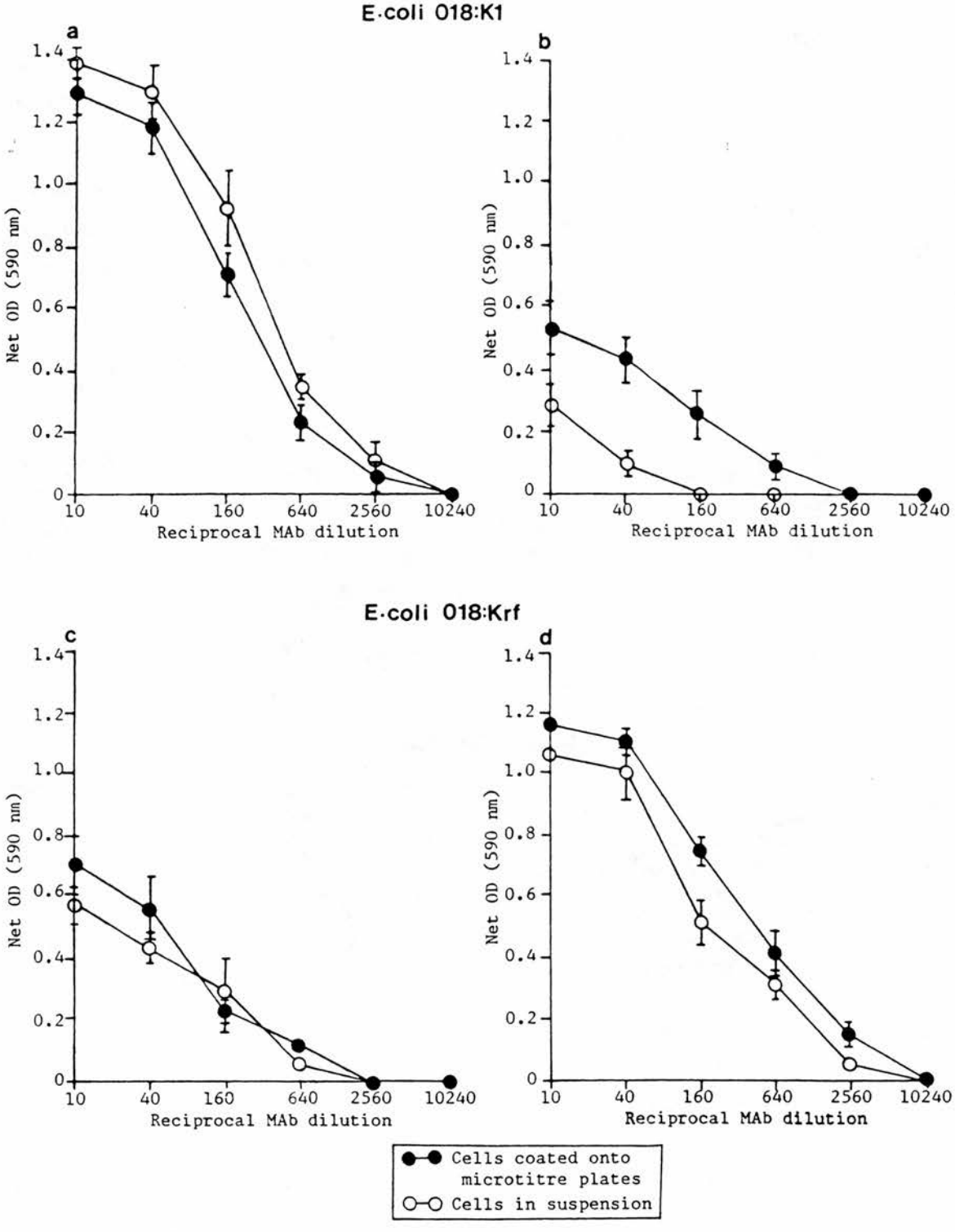


Figure 93. ELISA activity of O18, O-antigen specific MAb 184.2.5.5 (a & c) and core-specific MAb 43.27.11.2 (b & d) against whole cells of *E. coli* O18:K1 (a & b) and its rough mutant O18:Krf (c & d). The ELISA assays were performed against cells coated onto microtitre plates and cells in suspension. Bars represent the SD of OD values from three experiments each carried out in triplicate.

DISCUSSION

CHAPTER 1

CHARACTERIZATION OF ANTI-LIPOPOLYSACCHARIDE MONOCLONAL ANTIBODIES

Monoclonal antibodies recognizing the lipid A, core oligosaccharide and O-polysaccharide components of the LPS molecule were selected from an existing panel exceeding 200, produced from more than 30 fusions as part of a collaborative multicentre study with the ultimate aim of identifying an anti-LPS cross-reactive antibody for immunotherapy of Gram-negative septicaemia and septic shock. Although several show promise as possible immunotherapeutic agents, others have proved invaluable as probes for studying the expression of LPS and its detection.

Several MAbs (27.150.3, 27.193.3, 30.4.2.8, 43.11.5.1 and 43.27.11.2) demonstrated some degree of cross-reactivity in ELISA against heat-killed cell preparations of selected Gram-negative species, representing a number of different LPS-core structures. This cross-reactivity supports the idea that shared antigenic determinants are present in the LPS of different species. The inner core region of LPS is believed to exhibit a high degree of structural conservation among all Enterobacteriacae (Eskenazy *et al*, 1977; Jansson *et al*, 1981; Perez-Perez *et al*, 1986). Serial absorption studies were used to identify the region in the LPS core to which the antibodies were directed. Those MAbs showing some cross-reactivity were indeed directed against the conserved inner or intermediate core region of LPS: 27.193.3 lipid A; 43.27.11.2 Re ('anti-lipid A-KDO'); 43.11.5.1 and 27.150.3 Rc ('anti-heptose'). MAbs 27.193.3 and 43.27.11.2 reacted with the full panel of Gram-negative heat-killed cell preparations in ELISA, suggesting the

site it recognizes is present in all these species. However, preferential binding of MAbs 27.193.3 and 43.27.11.2 to the *E. coli* core R3 and R1 respectively, may indicate that their epitopes are relatively exposed in some core types and not in others, perhaps as a function of differences in core structure. In contrast, the Rc reactive MAbs 27.150.3 and 43.11.5.1 appeared to recognize epitopes present only on *E. coli* and *Salmonella*, two genera with similar core structure.

MAb 30.4.2.8, although demonstrating cross-reactivity against most heat-killed cell preparations, preferentially reacted against the smooth serotype of *E. coli* O18. Immunoblotting established an affinity for the O-polysaccharide of the O18 serotype (Figure 18b). Since the MAb was cloned from a fusion prepared after a vaccination procedure which included the immunogen lipid A, but no O18, O-antigen, it was considered unusual for the MAb to show serotype reactivity. A mix-up in cell lines with those of other fusions is a possibility which cannot be discounted. However, it is possible that the MAb is multispecific, binding to epitopes present on both lipid A and O-antigen LPS components. Indeed, chemical analysis of the O18, O-polysaccharide (Gupta *et al*, 1984; Jann & Jann, 1987) revealed the presence of glucosamine, a sugar also present in lipid A. Serial absorption (Figure 25) and inhibition studies (data not shown) demonstrated that 30.4.2.8 does have some affinity for lipid A, although incomplete absorption of MAb reactivity by *S. typhimurium* lipid A indicates that this affinity is rather weak.

The core-glycolipid reactive MAbs of 43.3.4.8, 43.5.1.4, 43.35.1.4 and 40.18.7.1 were restrictive in their specificities, preferentially binding to selected *E. coli* core types. Results indicated that these

MAbs recognize epitopes in the outer core hexose region, structurally representing the most variable part of the core (Lüderitz *et al*, 1982; Westphal *et al*, 1983; Perez-Perez *et al*, 1986). The induction of specific core or R antibodies is not uncommon, since the terminal sugar of rough mutant bacteria represents the immunodominant sugar (Brade *et al*, 1988). It is postulated that the host will be exposed to Gram-negative bacteria containing complete (Ra) core, LPS with S-LPS, stimulating the production of core 'type' specific antibody responses of limited cross-reactivity (Scott *et al*, 1990). Such antibodies may not be depressed during endotoxaemia while antibodies to the LPS inner core are depressed (Barclay *et al*, 1989). Failure to observe any reduction in ELISA activity for *E. coli* R1 reactive MAbs 43.3.4.8, 43.5.1.4 and 43.35.1.4 after serial absorption with lipid A and R chemotypes of *S. typhimurium* reflected differences in the outer core structure of *S. typhimurium* and *E. coli* R1. The corresponding absorption for MAb 40.18.7.1, reactive primarily against *E. coli* R2 and *Salmonella* strains, indicated specificity for the Rc chemotype. In contrast to other *E. coli* core types, R2 possesses an almost identical core structure to *Salmonella*, only differing in the nature of one hexose residue (Figure 5). Similarity in these core structures reflects this selective specificity of 40.18.7.1 for the intermediate /outer core region of both *E. coli* R2 and *Salmonella*.

The discrete core type specificity of many of these core-reactive MAbs represents an extremely valuable set of tools for studing the serology of the endotoxin core. Indeed, colleagues within the laboratory have used similar anti-LPS MAbs of limited cross-reactivity to assign core types to 80 *E. coli* clinical blood culture isolates, A.P. Gibb, G.R. Barclay, I.R. Poxton and F. Di Padova; unpublished data.

Immunoblot analysis of core-glycolipid reactive MAbs established that the majority reacted against unsubstituted core oligosaccharide. The epitopes recognized by these MAbs may represent the immunodominant terminal sugars of rough mutant bacteria which become unavailable when O-polysaccharide is linked. Alternatively, the presence of O-polysaccharide may induce a conformational change in the core structure, restricting epitope accessibility. Cross-reactive MAb 27.150.3 (Figure 17b) represented the only antibody investigated in this study which is reactive against the ladder pattern of unsubstituted core and core substituted with O-antigen.

The overall binding specificities of particular core-glycolipid reactive MAbs appeared similar in different assays. Yet, significant differences were noted in the recognition of certain antigens by particular MAbs. Examples included the reactivity of 43.27.11.2 and 27.193.3. Both MAbs showed broad cross-reactivity against heat-killed cells in ELISA, yet restricted specificity against *E. coli* expressing the R1 and R3 cores respectively in immunoblotting and flow cytometry. A comprehensive study by Aydintug *et al* (1989) established that in addition to epitope specificity, cross-reactivity of MAbs is a function of the method of preparation of bacterial antigens and the assay itself. The differing results between ELISA and immunoblotting implies: immunoblotting is less sensitive than ELISA, or differences in the physical presentation of LPSs between the assays results in antigenic alteration or differences in antigen accessibility. Aydintug *et al* (1989) attempted to increase the sensitivity on immunoblots using avidin-biotin reagents. As a result, the intensity of positive results increased, but the negative results were unchanged. The latter hypothesis is a more likely explanation for differences in results.

The amphipathic nature of the LPS molecule confers susceptibility to a variety of noncovalent interactions with, and conformational influences imparted by, its physical environment (Galanos & Lüderitz, 1984). Altered conformation of the molecule may stereochemically hide cross-reactive epitopes. Detergent used for electrophoresis would solubilize LPS to a monomeric form, possibly influencing antigenic activity.

The development of a proteinase K extracted LPS ELISA allowed the direct comparison of MAb specificity against similarly prepared antigens in ELISA and immunoblotting. The cross-reactivity of MAbs appeared greater in ELISA employing proteinase K extracted LPS than in immunoblotting. Indeed, binding specificities of MAbs in the proteinase K ELISA were generally similar to the heat-killed cell ELISA. Whilst ELISA is perhaps a more sensitive assay than conventional immunoblotting, LPS is probably displayed differently in the two assays, accounting for distinctive patterns of recognition by certain MAbs. Assay dependent differences in antibody specificity suggest the need for more than one assay when defining MAb specificity. This requirement is important for core- and lipid A-reactive antibodies because of the unique physicochemical and immunological properties of LPS (Pollack *et al*, 1989).

The extraction of LPS by the proteinase K digestion of cells for coating to ELISA plates established itself as a simple method, having the additional advantage of requiring only small culture volumes. The main concern of the extraction procedure was the nature of the digested product. Despite establishing optimal conditions for the efficient digestion of cells, microscopy confirmed the presence of cell fragments which were removed by centrifugation. Silver stain analysis demon-

strated that LPS was the only major macromolecular component of digested samples. Initial studies established the poor reproducibility of coating extracted R-LPS in particular, directly onto ELISA plates. Complexing LPS with polymyxin B gave stable binding of proteinase K extracted LPS to microplates. Polymyxin B is a small cyclic cationic polypeptide with a fatty acid side chain which probably binds predominantly to the inner core region of LPS through both hydrophobic and cationic interactions, to form a relatively stable complex (Morrison & Jacobs, 1976; Schindler & Osborn, 1979). The reproducibility of the LPS-polymyxin assay was better than LPS alone when results of initial and two repeat experiments were compared (Figure 29). Similar results were presented by Scott & Barclay (1987) who developed a method of complexing purified LPS to polymyxin B for the detection of anti-core-glycolipid IgG antibodies in blood donor sera. This procedure enhanced the antigenicity of the LPS, enabling detection of antibodies to R-LPS which were not detected in ELISA where purified R-LPS alone was used. Scott & Barclay (1987) proposed that polymyxin may stabilise the LPS structure, allowing the LPS to mimic its natural conformation where it is complexed to protein in the bacterial outer membrane (Costerton *et al*, 1974; Vaara & Hiroshi, 1984) or complexed to various host plasma constituents or cells (Ulevitch *et al*, 1981; Munford & Dietschy, 1985).

Inhibition of ELISA reactivity with different LPS antigen preparations, was performed to determine the antigenic configuration of proteinase K extracted LPS (complexed or uncomplexed) relative to other LPS preparations. The most efficient inhibitor of MAb ELISA activity was purified LPS complexed with polymyxin. The inhibitory effect of proteinase K extracted LPS (either complexed or uncomplexed) was similar, although generally lower than that of purified LPS-polymyxin

complexes, indicating good antigenic presentation. Failure of polymyxin to improve antigenicity significantly, when complexed with proteinase K extracted LPS, suggests natural accessibility of the uncomplexed form to antibodies. The optimal coating concentration of the complex was 1:10 dilution in coating buffer. Greater concentrations may present an excess of complex to binding sites on the solid phase, giving rise to unstable coating and loss of complex from the solid phase during assay.

Flow cytometry on whole intact bacteria indicated that the O-antigen of LPS restricted access of antibody to epitopes in the inner core region of the molecule in particular. The absence of O-antigen on rough mutants appeared to increase the availability of core LPS epitopes to antibodies, resulting in better binding than to smooth strains. The overall effect was striking MAb exclusivity, with the majority of core-specific MAbs showing either low or no reactivity against smooth bacteria. The core-reactive MAbs exhibiting the highest binding levels were specific for the more accessible outer core region. Thus, although there are shared antigenic determinants in the core-glycolipid region of LPS, they are not necessarily available for binding by antibody (Elkins & Metcalf, 1985; Gigliotti & Shenep, 1985). However, heat treating smooth bacteria prior to flow cytometric analysis significantly increased anti-core MAb binding. Conserved antigenic determinants that may be cryptic on intact bacteria may be unmasked only after heat treatment. Tsuchido *et al* (1985) proposed that heating may disrupt the integrity of the outer membrane and thereby increase permeability. Studies demonstrating extensive MAb cross-reactivity against heterologous intact bacteria (Nelles & Niswander, 1984; Miner *et al*, 1986; Baumgartner *et al*, 1987) may have been exaggerated on the

basis of using binding experiments employing boiled cells.

Flow cytometry has been used extensively to analyse eukaryotic cell populations (Muirhead *et al*, 1985), although its application to bacteria has remained limited. Several investigations have employed the technique to measure changes in bacterial protein or DNA content during cell growth (Steen & Boye, 1980; Boye *et al* 1983), and also to detect or identify bacteria in body fluids and environmental water supplies with varying success (Mansour *et al*, 1985; Tyndall *et al*, 1985; Robertson & Button, 1989). A recent study by Lutton *et al* (1991) also used flow cytometry to obtain a qualitative indication of epitope expression on different populations of *Bacteroides fragilis*. Flow cytometric analysis of the binding of anti-LPS MAbs to bacteria presented in this thesis was a successful application of this technology to bacteria. It offers a means of investigating surface properties of individual cells in large numbers with great speed and efficiency, whilst also analysing cells in their natural form, devoid of some of the potential distorting influences present in other methods. Despite the undoubted potential of flow cytometric analysis of bacteria and perhaps other prokaryotes, it remains largely unexplored and still in its infancy. Indeed, the only other known study applying the technique to analyse LPS-reactive MAbs to bacteria includes that of Evans *et al* (1990). Their work established only limited ability of all core- and lipid A-reactive antibodies to recognize corresponding epitopes on wild-type smooth bacteria.

Phillips *et al* (1987) centrifuged fluorescent labelled reaction mixtures through a sucrose solution as a means of removing unreacted antibody, thus reducing the antibody component of the fluorescent

signal without encouraging aggregation of bacteria. It was stressed that the potential of flow cytometry for studying antigen distribution in bacteria will only be fulfilled by accurately measuring single bacteria in a population, without confusion from aggregates. Clumps of cells were removed by the use of gates on the log forward angle light scatter signal for work presented in this thesis, whilst Lutton *et al* (1991) used mild sonication to break bacterial aggregates. In addition to encouraging aggregate formation, centrifugation washing during the preparation of bacterial samples for flow cytometric analysis has the added disadvantage of possible loss of bound antibody during the resuspension process. Consequently, a reduction in the intensity of fluorescent signal and perhaps the actual proportion of positive fluorescent cells in a population may result.

Concern regarding the possible effects of binding whole bacterial cells to a solid-phase matrix was highlighted in the analysis of the whole cell ELISA technique. A comparison of the binding characteristics of LPS reactive MAbs in ELISA, against coated untreated whole cells, and cells disrupted to different extents by heating and sonication, indicated that whole cells, whose core LPS epitopes were the least accessible, represented the most intact preparation. Studies using suspended whole cells revealed that although the outermost O-antigenic side chains were very inhibitory against homologous MAb binding (184.2.5.5), a very weak inhibitory effect was shown against inner core-reactive MAb 43.27.11.2. These results suggested possible differences in the presentation and accessibility of suspended and coated whole cells. An attempt to compare directly the binding of core-reactive MAbs to suspended and coated cells in ELISA, again indicated greater availability of epitopes for cells attached to the

solid-phase, due probably to their alteration during binding manipulations. In contrast, flow cytometric analysis under near physiological conditions, circumvents the problems of solid-phase induced antigen alteration. However, ELISA offers the advantages of speed, flexibility, quantitative accuracy and perhaps greater sensitivity.

Failure to demonstrate cross-reactivity of selected MAbs specific for the conserved regions of the LPS molecule in certain assays, highlights the ongoing controversy regarding the existence of broad cross-reactive core-glycolipid antibodies capable of binding to a variety of smooth strains. The use of different procedures for the characterization of MAbs by several investigators, has led to inadequate proof of MAb cross-reactivity, and emphasized the need for a reliable and reproducible *in vitro* technique for measuring reactivity of MAbs to heterologous Gram-negative bacteria and LPS. Heumann *et al* (1991) developed an ELISA employing LPS complexed with HDLP, a natural carrier of LPS *in vivo* (Ulevitch *et al*, 1981). Similar to LPS-polymyxin complexes, the LPS-HDLP complex is thought to result in a more physiologic presentation of the antigen, and to possess the additional advantage of reducing non-specific binding to LPS. Warren *et al* (1991) developed a fluid-phase radioimmunoassay, performed in the presence of normal serum to facilitate the detection of IgG in antisera that binds to heterologous S-LPS. The assay avoided binding of antigen to a solid-phase and was considered appropriate for the accurate analysis of anti-LPS MAbs.

It was apparent from a variety of selected MAbs with specificities for different epitopes in the core-glycolipid region of LPS that most of

these MAbs were more restrictive in their specificity than cross-reactive. Results emphasize the immunochemical complexity of the core-lipid A region of enterobacterial LPS. Despite acknowledged structural conservation among core-glycolipids from different Gram-negative bacteria, substantial microheterogeneity is likely to occur which will affect the degree of MAb cross-reactivity (Pollack *et al*, 1989). The fine-binding specificity of MAbs may not allow for the desired broad-spectrum protection against infections with Gram-negative bacteria. Indeed, limited binding specificities of anti-LPS MAbs has implications which question whether a therapeutic product should be a single antibody, or a cocktail of antibodies covering the spectrum of core-glycolipid epitopes.

CHAPTER 2

ENVIRONMENTAL MODULATION OF *ESCHERICHIA COLI* LIPOPOLYSACCHARIDE EXPRESSION

2.1 EFFECTS OF NUTRIENT GROWTH CONDITIONS

The environmental modulation of cell surface components of both Gram-positive and Gram-negative bacteria has been intensively investigated (Brown & Williams, 1985). Among the environmental factors that commonly influence the properties of microbial cells, the availability of essential nutrients assumes particular importance (Harder & Dijkhuizen, 1983). Numerous studies have determined the binding of anti-LPS MAbs to purified LPS or intact bacteria grown under laboratory conditions not associated with the *in vivo* environment. This study examined the effects of a number of nutrient growth conditions relating to those *in vivo* on the accessibility and expression of LPS antigens on bacteria using MAbs.

The expression of *E. coli* core LPS was increased when grown in modifications of Malka minimal medium, containing depleted Mg^{2+} concentrations (between no added magnesium salt and 1.7 mmol L^{-1}, cf. 0.65-1.0 mmol L^{-1} in serum). This was reflected in greater binding of anti-core MAbs using the techniques of immunoblotting, whole cell ELISA and flow cytometry. Magnesium is important in maintaining the stability of the structural arrangement of larger molecules such as LPS within the outer membrane of Gram-negative bacteria (Costerton *et al*, 1974). Cell walls of magnesium-limited cells of *Bacillus subtilis* showed an increased Mg^{2+} binding affinity over magnesium-sufficient cells (Meers & Tempest, 1968). Thus certain organisms may respond to

magnesium limitation by improving their ability to bind the ions by increasing negatively charged cell surface components such as LPS, therefore maintaining the structural integrity of the bacteria and its functional ability to grow. Using both batch and continuous culture, Day & Marceau-Day (1982) reported gross changes in both the amount of LPS produced per cell and the composition of the LPS in response to changes in Mg^{2+} concentration. Compositional changes in the molecule were also reflected as functional alteration in the LPS. Data indicated a change in core size of LPS relative to the O-antigen, suggesting the possibility of magnesium having a regulatory role on one or more of the LPS biosynthetic enzymes. Similarly, an increased production of low molecular mass LPS was shown for *E. coli* grown at intermediate growth rates under magnesium limitation (Dodds *et al*, 1987), whilst magnesium depleted cells of *P. aeruginosa* produced more LPS as quantified by changes in KDO content than glucose depleted cells (Gilbert & Brown, 1978).

Flow cytometric analysis identified bacterial subpopulations exhibiting distinct patterns of MAb reactivity. *E. coli* O18:K1 possessed two subpopulations of cells when grown under magnesium depleted conditions and probed with an O-antigen specific MAb (Figure 67a). Reasons for this variability of MAb binding within a population of cells include: differences in the accessibility and composition of LPS; capsule expression (shown to be inhibited under these conditions by Taylor *et al*, 1981b); or morphological heterogeneity of bacteria grown under magnesium depletion. The ability of flow cytometry to analyse individual cells also lent itself to sorting bacteria on the basis of differential MAb binding due to variations in antigenic surface structure. The instability of sorted phenotypic variants (Figure 69)

suggests that the basis of any difference between subpopulations is not due to a stable genetic alteration. The phenomenon of antigenic phase variation was demonstrated for strains of *H. influenzae*, capable of expressing antigenic changes in LPS by spontaneous loss or acquisition of O-antigen specific MAb reactivity (Kimura & Hansen, 1986). Genetic variation at a chromosome locus responsible for the expression of LPS-associated epitopes was in part responsible for the observed variation in *H. influenzae* LPS. Flow cytometric analysis also identified two distinct phenotypic variants showing differences in reactivity with a core-specific MAb (Evans *et al*, 1990). Increased identification of antigenic variation among assumedly homogeneous bacterial populations has important implications regarding the potential for bacteria to evade LPS-specific host defence mechanisms.

The changes in LPS composition in response to magnesium depletion could also be expected to influence the functioning of the cell envelope. Magnesium concentration affected the capacity of the LPS molecule to bind charged molecules such as lysozyme and gentamicin (Day & Marceau-Day, 1982) which in turn may be reflected in other such physiological changes as antibiotic resistance. In terms of the cell however, increased LPS production appears to offset the decreased binding of these molecules. Changes in the envelope structure and cation content of magnesium depleted cells have been associated with increased resistance to polymyxin and EDTA (Finch & Brown, 1975; Kenward *et al*, 1979). Polymyxin is bactericidal through its interaction with the outer membrane, which results in disruption of that membrane with a loss in permeability function (Teuber, 1974). Since polymyxin appears to compete with Mg^{2+} and Ca^{2+} for binding sites within the outer membrane, changes in the LPS and phospholipid composition of the outer

membrane reduce the number of binding sites and render the membrane less permeable to polymyxin (Gilleland, 1988).

Detailed chemical analysis of LPS samples involving nuclear magnetic resonance studies was restricted to LPS isolated from nutrient broth and magnesium depleted conditions (data not presented). The structure of the O18 O-antigen for both capsulate and non-capsulate strains resembled that of other published data (Gupta *et al*, 1984; Jann & Jann, 1987). Magnesium depleted *E. coli* O18 LPS samples contained reduced O-antigen, whilst other complex data indicated that rhamnose, a constituent of O18 O-antigen was replaced by an as yet unidentified sugar. More detailed studies concerning the phenotypic heterogeneity of the chemical composition of LPS structures is clearly needed, complementing analysis of LPS expression as presented in this thesis.

The low availability of iron is acknowledged as a key determinant of virulence (Griffiths, 1987). Initial investigations showed little effect of iron depletion on LPS expression as determined by silver staining and immunoblotting. However, increasing the degree of iron deprivation using the iron chelator 2,2' dipyridyl, led to a greater expression of rough core at the expense of high molecular mass O-polysaccharide LPS, possibly reflecting a regulatory effect of iron on LPS biosynthetic enzymes. Alternatively, reduced growth rates under these conditions may also have produced this effect. Growth rate per-se can have significant effects on the composition of the cell envelope and LPS (Gilbert & Brown, 1978; Kenward & Brown, 1978). In a chemostat culture at high dilution rate, *E. coli* O15:H7 cells produced more low molecular mass LPS than high molecular mass LPS, compared to slow growing or batch cultured cells (Dodds *et al*, 1987). Since variables

other than nutrient limitation influence the expression of LPS, it is acknowledged that in using the batch culture model, the significance of altering one variable cannot be divorced with certainty from the influence of others. Only chemostat continuous culture offers the facility to examine the effects of a single environmental variable with all other factors remaining constant.

Although changes in the expression of LPS have been illustrated, growth under stress conditions such as iron and magnesium depletion are likely to alter the structure and composition of other components of the outer membrane. Thus, LPS core determinants normally showing limited accessibility may become better exposed on the cell surface. Whilst this study attempted to assess the binding of anti-LPS MAbs to whole cells, release of membrane fragments from cells must also be considered. Indeed, variations in growth conditions could alter the extent and nature of the excretion of such fragments (Hoekstra *et al*, 1976). Growth conditions showing changes in binding of anti-LPS MAbs to assumedly whole cells in ELISA and flow cytometry were however reproduced in immunogold electron microscopy studies, which demonstrated binding to whole bacterial thin sections.

An increased expression and accessibility of core LPS was shown for smooth *E. coli* grown in heat-inactivated sheep serum. Immunoblotting, ELISA and flow cytometry all showed significant increases in binding of anti-core MAbs despite an apparent increased production of O-antigen as revealed by silver staining. Growth under nutrient limiting conditions of serum, including iron and magnesium depletion may have been responsible for changes in the expression of LPS components and exposing previously inaccessible epitopes. The presence of any natural

anti-LPS antibodies within the serum may have also caused a false impression of MAb binding. However, since MAbs were raised in a different species to the serum used, antibody conjugate should recognize only mouse MAbs, and removing any anti-LPS antibodies from the serum by absorption had no effect on results. Frank *et al* (1987) postulated that natural antibodies against O-antigen permitted access of core-glycolipid antibodies. Absorption of serum antibodies with either *E. coli* O18 or unrelated *E. coli* O6:K5 showed no difference in anti-core MAb binding. Other investigators have proposed that 'enzymatic factors' present in serum are able to unmask the conserved rough antigenic structures of smooth Gram-negative bacteria, making these sites available for antibody binding (Chedid *et al*, 1968). It is possible that core-reactive antibodies could bind bacteria after partial disruption of the cells by other host defence mechanisms. For example, the iron-binding proteins of lactoferrin and transferrin may have additional antimicrobial effects. Recent work suggested that these proteins can directly damage the outer membrane of Gram-negative bacteria in a manner similar to the synthetic chelator EDTA (Ellison *et al*, 1988; 1990). Furthermore, it is known that the physiochemical configuration of LPS is considerably altered in the presence of serum or plasma (Warren *et al*, 1991), perhaps promoting enhanced anti-core MAb binding.

Greater expression of O-antigen from cells grown with serum at each stage of the growth cycle compared to nutrient broth conditions (Figure 65), may reflect the selection of factors which increase resistance to the bactericidal activity of serum (Taylor & Robinson, 1980; Goldman *et al*, 1984; Porat *et al*, 1987). The bactericidal activity of complement was highlighted when comparing the growth kinetics of *E. coli* O18:K1

and its non-capsulate isogenic mutant O18:K1$^-$ in heat-inactivated sheep serum and normal sheep serum. Both growth rate and final cell numbers were reduced when *E. coli* O18:K1$^-$ in particular were grown in untreated serum.

The progressively increased substitution of core LPS by O-antigen throughout the growth curve, underlined the importance of growth phase on LPS expression in addition to other factors, including nutrient growth conditions. These results were related to the finding that whole cell binding of core-reactive antibodies were maximal during the early log phase of bacterial growth, diminishing markedly thereafter (McCallus & Norcross, 1987; Pollack *et al*, 1987). Thus, intermediate or incomplete forms of the LPS molecule with relatively exposed core determinants may be produced early in the normal bacterial growth curve. McCallus & Norcross (1987) described how this phenomenon may be used as a basis for speculation that active immunization, whereby the antibody is present before the invading bacteria, may be more effective than passive administration of specific antibodies at a time when the bacteria are more mature. Growth of bacteria to the early stationary phase under all nutrient conditions was an attempt to eliminate the effects of isolating organisms from different growth phases on LPS composition.

Although capsule plays an important role in the serum resistance of many bacteria (Leying *et al*, 1990), it has been reported not to provide a barrier function for binding of anti-O antibodies to *Klebsiella* species (Williams *et al*, 1988) and some *E. coli* including O-serotype O18 (Cross *et al*, 1986). Indeed, binding of the anti-O18 MAb to K1 capsulate and non-capsulate strains of *E. coli* O18 showed no effect due

to the presence of capsule. However, greater binding of anti-core MAbs to the non-capsulate strain indicates capsules may function as a barrier for the penetration of anti-core LPS antibodies. The ability of both O-antigen and core-reactive MAbs to bind capsulate and non-capsulate strains of *E. coli* O18:K1 and O18:K5 were compared (data not presented). Both K1 and K5 capsules again showed no barrier function against O-antigen specific MAbs, whilst capsule presence appeared to impede anti-core MAb binding. However, *E. coli* O18:K5 and its non-capsulate isogenic mutant showed lower binding levels of both anti-core and O-antigenic MAbs against equivalent capsulate and non-capsulate strains of O18:K1. When LPS size distribution were assessed using PAGE, capsulate and non-capsulate mutants of both O18:K1 and O18:K5 were identical. These findings suggest the behaviour of the same O-phenotype may alter depending upon its associated K types. Kim *et al* (1986) demonstrated differences between the ability of K1 and K5 capsules in preventing anti-LPS antibody from mediating the killing of *E. coli* bacteria. A neonatal rat model of infection showed that the O18:K5 capsule conferred resistance against an anti-LPS MAb by preventing interaction with its target. In contrast, this same MAb protected these animals from lethal infection with an *E. coli* O18:K1. This data highlighted differences between the two capsules in their ability to prevent anti-LPS antibodies from mediating the destruction of the bacterium, possibly due to differences in their permeability to opsonins or their distribution on the bacterial cell wall (Cross, 1990). Further work is required to determine the effect of growth conditions on capsule expression, their influence on penetration of antibodies to cell surface components, and subsequent opsonisation and phagocytosis of capsulate bacteria.

Growth under nitrogen deficient/high carbon conditions has previously shown to promote capsule formation (Sutherland & Wilkinson, 1965). Reduced binding of anti-core MAbs to both capsulate and non-capsulate bacteria grown under these conditions suggested another factor, other than capsule exerting an influence. Silver staining and immunoblotting revealed an increase in the fraction of LPS species containing O-antigen, and an increase in the length of the attached O-antigen polymers. This suggested that the polymerization of O-antigen and its transfer to core-glycolipid may increase relative to synthesis of core-glycolipid in this nutritionally rich, high carbon medium. Since O-antigen is known to reduce accessibility of antibody to core LPS (Nelles & Niswander, 1984; Gigliotti & Shenep, 1985), a change in both its production and possible arrangement may have strengthened this effect.

The influence of growth *in vivo* on LPS composition and binding of LPS reactive MAbs to whole cells was attempted in this study. Great emphasis has been placed on the importance of studying the *in vivo* characteristics of bacterial envelopes which will differ markedly in physiology, biochemistry and immunogenicity from standard *in vitro* conditions (Williams, 1988; Smith, 1990). Indeed, putative determinants of pathogenicity indicated by *in vitro* studies may not be produced *in vivo*, whilst virulence determinants found *in vivo* may be missed under certain *in vitro* conditions (Smith, 1990). Animal models employing chamber implant devices have been used to mimic the *in vivo* environment and permit the recovery of sufficient quantities of bacteria for analysis. Permeable to humoral factors and antibiotics, and having the advantage of localising the infection, they do however prevent the access of host cellular defences (Williams, 1988).

Although not precisely mimicking infection, the model can be used to demonstrate bacterial cell properties while cells are growing on available nutrients *in vivo*. The recent development of a novel titanium chamber implant enjoys the advantages of a sampling portal located subcutaneously; is reusable; easily constructed and avoids inherent leakage problems of the traditional self-constructed plastic chambers used in this thesis (Pike *et al*, 1991). The mucin-haematin mouse septicaemia model was used to mimic a true infection, starting with inocula in the range of those found in human septicaemia, and characterized by *in vivo* multiplication of the bacteria.

The major difference noted for smooth *E. coli* strains grown *in vivo* when compared with its *in vitro* grown counterpart related to the increased length of the LPS O-antigen chain (Figures 72 and 75). The heterogeneity of LPS chain length implies that the polysaccharide chain formation is not tightly controlled, and thus readily modulated by changes in the growth environment. Several possible environmental effects on O-antigen chain formation by wild-type strains have been identified, including carbon supply, growth rate and temperature (McConnell & Wright, 1979; Taylor *et al*, 1981b). The susceptibility of Gram-negative bacteria to phagocytosis and to the bactericidal action of complement is thought, at least in part, to be determined by the properties of the LPS-polysaccharide chain (Goldman & Leive, 1980; Taylor, 1983). Therefore, the increase in chain length for *in vivo* grown *E. coli* may enhance its ability to resist multiple host defence systems. *In vitro* studies by Weiss *et al* (1986) demonstrated a close correlation between changes in the LPS chain length induced under different growth conditions and sensitivity to bactericidal/permeability-increasing protein. Changes in the LPS composition of *N.*

gonorrhoeae and *P. aeruginosa* cells grown *in vivo* in chamber implants have also been reported (Demarco de Hormaeche *et al*, 1988; Kelly *et al*, 1989). The latter study showed that compared with growth *in vitro, in vivo* grown *P. aeruginosa* cells lacked a series of high molecular mass O-antigen bands and gained a new series of lower molecular mass bands. This phenotypic alteration in LPS composition had no effect on bacterial serum resistance.

Analysis of the binding of LPS reactive MAbs to *in vivo* grown whole cells was limited by high background signals, probably caused by natural antibodies against cell surface components and binding of host proteins to bacteria. However, ELISA inhibition studies indicated that core epitopes, relatively inaccessible on *in vitro* nutrient broth grown bacteria were more exposed on *in vivo* grown bacteria, despite a longer polysaccharide chain length. These results lend support to the theory that anti-core-glycolipid antibodies bind to intact organisms under certain conditions. Further studies on the bacterial growth characteristics and phenotypic expression of LPS on bacteria grown *in vivo* are however still required, to establish clearly the binding capacity of core-glycolipid MAbs on intact bacteria.

The techniques of immunoblotting and silver staining were invaluable for analysing the LPS composition of cells grown under different nutrient conditions. However, concern exists regarding the accuracy of using optical density of washed bacteria to quantify the amount of loaded LPS. Absorbances (A_{525}) of between 0.5 and 0.6 have been shown to produce no significant differences in staining intensity, suggesting changes that do occur, result largely from alteration in LPS expression (Dr D. Smith, pers. comm.). Inherent variability in the staining

intensity of different silver stained gels, highlighted the need to restrict comparison of LPS profiles to the same gel. The greater resolution provided by gradient gels indicated what was previously thought to represent a single unsubstituted LPS band may actually represent several distinct bands (Figure 56). Alternatively these may represent aggregates of LPS molecules.

The presence of small amounts of S-LPS on the *E. coli* rough mutant O18:Krf was revealed by immunoblotting with O18, O-antigen specific MAb 184.2.5.5. This may be a result of partial reversion to the parent strain or the presence of small amounts of S-LPS in native O18:Krf cell walls. Failure of silver staining to detect this 'leakage' may reflect the relative insensitivity of the technique in detecting small amounts of O-antigen LPS. Fomsgaard *et al* (1990) however, established that LPS fractions containing a low number of fatty acids are washed out of the gel during the initial fixation stage. Omitting this step and increasing LPS oxidation time restored the ability to detect previously unstained LPS fractions.

This study has underlined that changes in the growth environment can lead to variation in LPS expression. Presumably the amount of LPS is a dynamic variable controlled by the bacterium to maintain fixed parameters such as surface charge. These and other outer membrane alterations allow the cell to adjust to changes in its surroundings, enhancing its survival in that particular environment. The diversity of phenotypic responses to environmental conditions by individual bacteria, suggests selective expression of a microbe's genome. Implicit in this is the role of DNA supercoiling and its involvement in the environmental regulation of gene expression, reviewed by Dorman

(1991).

Variation in the expression of LPS and other outer membrane components in response to altered growth conditions, has both important implications and applications. *In vitro* and *in vivo* studies demonstrating greater exposure and expression of specific LPS epitopes on the bacterial cell surface may increase the potential of immunotherapeutic MAbs targeted towards conserved regions of the LPS molecule. The possibility of similar changes in LPS expression taking place during cases of septicaemia must be viewed with caution but cannot be excluded. LPS *in vivo* may exist, bound to the bacterium or host components such as HDLP and endothelial surfaces, or be 'free' in micelles, all of which can also reasonably affect the availability of core structure to antibody. Efforts to stimulate the production of MAbs or high titre antisera against conserved LPS epitopes, should also consider the possibility of increasing antigenicity by controlling the environment.

2.2 EFFECTS OF SUBINHIBITORY CONCENTRATIONS OF ANTIBIOTICS

In contrast to the considerable accumulation of information on the effects of antibiotics on primary targets such as peptidoglycan, protein synthesis or DNA supercoiling, it is only relatively recently that attention has focused on the multiple secondary effects of antibiotics on the cell, especially on its envelope. Such secondary effects are of interest, particularly when sub-MICs of antibiotics, which can appear in body fluids and tissues during the course of chemotherapy, are involved. Growth in the presence of sub-MICs of antibiotics has been shown to affect the synthesis of a variety of virulence factors, many of which may help the host to eradicate

bacteria (Shibl, 1983; Gemmell, 1987).

Several reports have shown that inhibition of peptidoglycan can influence LPS synthesis. Taylor *et al* (1982) described a reduction in the length of the O-antigen side chain of *E. coli* following growth in the presence of sub-MICs of mecillinam and a concomitant increase in sensitivity to serum complement. A similar loss of O-antigen was reported by Overbeek *et al* (1987) when *E. coli* O111 was grown in the presence of sub-MICs of carumonam, exposing core-glycolipid. In contrast, a stimulation of LPS incorporation into the outer membrane has been demonstrated for certain β-lactam antibiotics (Essig *et al*, 1982). The major change in LPS expression shown in this thesis after growth in sub-MICs of antibiotics was caused by chloramphenicol. A relatively small reduction in the length of O-antigen side chain and/or greater expression of lower molecular mass LPS bands was observed. This effect appeared to be dependent on both the *E. coli* strain and concentration of antibiotic. Sub-MICs of chloramphenicol may affect the production of enzymes involved in the polymerization of the O-side chains. An increase in the length of the O-side chains after treatment with 1:2 sub-MIC of ciprofloxacin may reflect the antibiotic's mode of action. Interference with the enzymatic activity of the DNA-gyrase may affect the expression of pathogenic determinants, including LPS. Alternatively, other factors including a reduction in growth rate in the presence of ciprofloxacin may have also increased the chain length.

This study demonstrated that sub-MICs of various antibiotics significantly enhanced the binding of core-reactive murine MAbs to a number of smooth *E. coli* strains. Failure to demonstrate any significant differences in LPS or protein composition of the bacterial cell wall by

PAGE for most antibiotics, indicates that major qualitative differences in cell wall composition are not responsible for the increased anti-core LPS MAb binding. However, alteration in LPS composition for some chloramphenicol treated *E. coli* strains may have contributed towards the increased exposure of core epitopes.

Certain antibiotics, present at subinhibitory levels, are known to render capsulate strains of Gram-negative bacteria more susceptible to opsonophagocytosis and to the bactericidal activity of human serum (Taylor *et al*, 1982; Williams, 1987; Veringa *et al*, 1988; Raponi *et al*, 1990). The direct influence of antibiotics on the production of capsules appears to be responsible for this effect, although at present there appears to be no known common mechanism to explain the influence of antibiotics on capsule formation. However, the synthesis of peptidoglycan, LPS and exopolysaccharide is coordinately regulated with cell growth and division, and certain sugar nucleotide precursors are common to each pathway. The translocation of precursors through the cytoplasmic membrane is also mediated via a common carrier, the undecaprenyl phosphate lipid intermediate (Troy *et al*, 1971). Thus, interference with peptidoglycan by certain antibiotics may indirectly affect exopolysaccharide production by a feedback mechanism on the lipid intermediate or by the accumulation of sugar nucleotides (Kadurugamuwa *et al*, 1985a). Whitfield *et al* (1984) also demonstrated that the initiation of K1 capsule appearance was dependent on protein synthesis, and the addition of chloramphenicol before temperature upshift prevented any expression of the K1 antigen. Chloramphenicol appears to affect a protein in the translocation of the polysaccharide across the cytoplasmic membrane (Kröncke *et al*, 1990b). Ciprofloxacin has also been shown to inhibit K1 production in a dose-dependent

manner, although the link between the antibody's primary mechanism of action and secondary effects is unknown (Suerbaum *et al*, 1987).

Growth of a capsulated strain of *K. pneumoniae* in sub-MIC of antibiotics reduced expression of capsule whilst increasing the exposure of several outer membrane proteins (Kadurugamuwa 1985a & b). Considering earlier studies demonstrating the role of capsule in masking inner core-glycolipid epitopes of *E. coli* strains, it is feasible that a similar loss of capsule after sub-MIC treatment may equally enhance binding of core-reactive MAbs. Similar increases in binding of core-reactive MAbs to antibiotic treated cells of capsulate *E. coli* O18:K1 and its non-capsulate isogenic mutant, indicate that any reduction in capsule expression played only a minor role in increasing the exposure of core epitopes. In contrast, observations by Overbeek *et al* (1989) suggested that the retention of some capsule actually limited the increased accessibility of deeper situated antigenic epitopes compared to non-capsulate strains. This effect was dependent on the relative thickness of the capsule. Variability among different *E. coli* strains of the effect of antibiotics on the binding of anti-LPS MAbs observed in this thesis, is perhaps a combination of factors, including type and thickness of capsule, O-serotype, and the ability of the antibiotic to permeate the outer membrane. Similarly, the relative increase in MAb binding between those directed towards either inner or outer core LPS epitopes also varied amongst *E. coli* strains, again perhaps reflecting differences in the ability of strains to mask specific epitopes after antibiotic treatment. The importance of bacterial strain was also highlighted by Taylor *et al* (1981a) who demonstrated variation in serum susceptibility of *E. coli* treated with mecillinam.

The ability of sub-MICs of various, unrelated antibiotics to increase the exposure of relatively inaccessible epitopes is perhaps mainly attributable to their modes of action. β-lactam antibiotics such as ampicillin directly affect the synthesis of the bacterial cell wall by interfering with the cross-linking of the peptidoglycan backbone (Atkinson & Amaral, 1982). Ciprofloxacin affects the DNA synthesis of bacteria (Wolfson & Hooper, 1985) which may cause several different metabolic and structural disorders. An enhanced binding of core-reactive MAbs to bacteria, grown in the presence of either antibiotic, could therefore be explained by a disturbed cell wall composition which unmasks relatively inaccessible R-LPS determinants. Cell elongation induced by both ampicillin and ciprofloxacin may also impair the integrity of the cell wall, permitting greater accessibility of antibody to cell surface antigens such as LPS. Sub-MICs of protein synthesis inhibitors, including chloramphenicol and gentamicin could feasibly impair synthesis of enzymes that are involved in cell wall synthesis, exposing inner core structures and subsequent enhanced binding of MAbs. Although traditionally gentamicin has been considered an inhibitor of protein synthesis, Martin & Beveridge (1986) also demonstrated an outer membrane destabilizing mechanism for gentamicin to increase penetration of the antibiotic. Peterson *et al* (1985) proposed that polycationic antibiotics such as gentamicin disrupt the LPS-Mg^{2+} cross bridges, rearranges its packaging, resulting in the formation of 'cracks' in the membrane structure. However, observations made in this study indicate that of those antibiotics used, gentamicin had the least effect on anti-LPS MAb binding. Despite this, Artenstein & Cross (1989) recently reported a direct endotoxin-neutralizing effect of aminoglycosides. Considering that many of the sequelae of sepsis may be related to endotoxin release after bacterial lysis, an agent

that directly inhibits LPS may be a vital component of an antimicrobial regimen.

Further evidence of greater accessibility of LPS epitopes following antibiotic treatment was highlighted in an investigation using HA-1A, a human IgM MAb recently licenced for therapeutic use against septic shock (Bogard & Siegel, 1991). These authors demonstrated a dramatic increase in the expression of its epitope on the lipid A domain of endotoxin, after treatment of bacteria with cell wall active antibiotics. In addition, the immunization of mice with ciprofloxacin-treated bacteria has been shown to enhance protection against a challenge with homologous and heterologous untreated bacteria, thought to relate to a significant increase in anti-LPS antibody titres (Raponi *et al*, 1991). These results indicated that host defences against Gram-negative bacteria may be influenced not only by a direct effect of antibiotics on the bacteria, but also an indirect effect of antibiotics on host defences by enhancing production of antibodies against untreated bacteria.

Data presented in this study lends additional support to the growing body of evidence suggesting possible clinical applications of sub-MICs of antibiotics. Possible synergism between antibodies and antibiotics presents intriguing possibilities surrounding both the prevention and treatment of septicaemia and septic shock in immunocompetent as well as immunocompromised hosts, recently reviewed by Overbeek & Veringa (1991). The potential of antibiotics to improve the exposure of conserved LPS epitopes targeted by immunotherapeutic MAbs, by overcoming the masking effect of overlying O-antigen in particular is likely to improve the clinical efficacy of such MAbs. The exposure of deeper

antigenic determinants by sub-MICs of certain antibiotics has the additional advantage of influencing host defences as previously described, including the promotion of opsonization by serum complement or antibodies, thus enhancing phagocytosis.

The *in vivo* relevance of this, and other studies concerning the effect of sub-MICs of antibiotics needs to be clearly established. Most investigations have been staged *in vitro*, quite different from the *in vivo* environment where nutrient limitation and presentation of the LPS molecule will also profoundly influence its susceptibility to both host defences and therapeutic agents (see section 2.1). The *in vitro* situation also allows growth of bacteria in a given concentration of antibiotic, whilst the *in vivo* concentration will be determined by body sites and a human's capacity to metabolize drugs. Limited *in vivo* studies however, indicate a possible decrease in mortality from Gram-negative septicaemia using combination therapy. Young *et al* (1989) studying mice challenged with *P. aeruginosa* demonstrated protection only when anti-lipid A MAbs were given in combination with certain antibiotics. Similarly, protection of leukopenic rats from lethal infection with *P. aeruginosa* was thought to be related to the synergistic action of suboptimal doses of ciprofloxacin and an anti-LPS MAb (Collins *et al*, 1989). However, only through additional studies will more insight be gained into the *in vivo* relevance of *in vitro* studies.

In conclusion, whilst much interest is focused on developing cross-reactive LPS MAbs for treating and preventing Gram-negative septicaemia and endotoxaemia, appreciation of the potential of additional therapy with appropriate antimicrobials and dosages is clearly indicated. Consideration must also be given to the standardization of

antimicrobial treatment when testing the efficacy of immunotherapeutic MAbs in clinical trials.

CHAPTER 3

DEVELOPMENT OF A MONOCLONAL ANTIBODY IMMUNOASSAY FOR THE DETECTION OF *ESCHERICHIA COLI* LIPOPOLYSACCHARIDE

The LAL assay is the classic method for detection of endotoxin in biological fluids. Despite high sensitivity, the usefulness of the *Limulus* method is, however, somewhat limited by non-specific activators and inhibitors of LAL (Cohen & McConnell, 1984; Obayashi *et al*, 1986). In addition, the LAL assay does not allow discrimination of different types of endotoxin, making the assay vulnerable to trace amounts of contamination involving endotoxins from other bacteria. Furthermore, endotoxins from different bacterial species exhibit significant differences in their relative abilities to activate LAL (Friberger *et al*, 1987).

The purpose of this study was to develop and evaluate a MAb based enzyme immunoassay for the detection of specific *E. coli* LPS core- and O-types in the serum of septic patients. The ability to detect specific endotoxins in body fluids would have numerous applications for patients with endotoxaemia. Classically, LPS is responsible for shock accompanying Gram-negative bacterial sepsis, although much debate exists concerning the role of endotoxins originating from the gut in addition to LPS from original infective organisms. The ability to monitor septic patients for specific endotoxins would help establish any relationship between detectable LPS and bacteraemic culture isolate. Results would have obvious implications for the potential of immunotherapeutic MAbs. The efficacy of MAbs targeted against the LPS of commonly isolated bacteria such as *E. coli*, would be severely

limited where LPS originates primarily from the gut. The specific detection of endotoxin in patients may also facilitate the selection of suitable therapeutic anti-LPS MAbs, possibly including cocktails of antibodies with specificity for accessible core- and O-antigenic epitopes.

There has been increasing interest in the development of assays capable of detecting specific LPSs. LPS is specific, highly immunogenic and easily released into the surrounding medium (Cadieux *et al*, 1983; Kusama, 1983) and therefore considered an appropriate antigen for the detection of certain Gram-negative bacteria. Enzyme immunoassays have been developed for the detection of LPS or related LOS from a variety of microorganisms including: *P. aeruginosa* for the early identification of respiratory colonization by the organism (Nelson, 1990); *H. influenzae* for the diagnosis of meningitis caused by this organism (Mertsola *et al*, 1989; 1990); *Brucella abortus* for the diagnosis of brucellosis (Perera *et al*, 1983; Limet *et al*, 1988) and *Salmonella* species for the diagnosis of *Salmonella* infection (Araj & Das Chugh, 1987; Lim, 1990 & Luk & Lindberg, 1991).

The use of MAbs in ELISA overcomes the problems encountered by using a polyclonal antibody. MAbs can be supplied in almost unlimited amounts once the hybridoma cell line is established; problems arising from batch variations are avoided when MAbs are used; MAbs generally show higher specificity since each molecule of antibody is directed at the antigen in question, and high concentrations of specific antibody can be obtained (Yolken, 1982; Harlow & Lane, 1988).

There are a number of ways in which enzyme immunoassay systems can be

formulated to measure infectious agents directly in body fluids (Yolken, 1982). In this particular study MAb based double antibody sandwich ELISA systems were developed for the detection of *E. coli* LPS core types R1-R4, specific *E. coli* LPS core types of either R1 or R3 and the *E. coli* 018 O-antigen in dilution buffer and in spiked serum. Bio-MAbs were used in the assays developed. Biotin labelling of antibodies is a simple mild reaction, allowing antibodies to retain full biological activity and giving high labelling efficiency. The stable monomeric conjugates also enjoy a number of advantages for use in enzyme immunoassays. A very high affinity between biotin and avidin provides stable complexes and has the potential for amplification at the level of co-factor enzyme interaction (Guesdon *et al*, 1979).

Biotin/MAb ratios of 1:1, 2:1 and 4:1 all yielded usable reagents and corresponds to other reports (Kendall *et al*, 1983; Yolken *et al*, 1983). Since one molecule of streptavidin can efficiently bind four molecules of biotin, multiple avidin-biotin interactions can be achieved, offering the possibility of increased sensitivity (Yolken, 1982). In this study the most efficient immunoassay system was one employing streptavidin labelled alkaline phosphatase to bind to the bio-MAbs. The use of streptavidin-biotin-alkaline phosphatase complexes is theoretically more sensitive, since it employs a second amplification step where each 'bridging' streptavidin molecule can bind three biotinylated alkaline phosphatase molecules. However, in this study, whilst showing slightly higher sensitivity, background signals were unacceptably high. Although biotin-streptavidin reagents are non-sticky in nature (Kendall *et al*, 1983) it is possible that bad backgrounds may reflect the sticky nature of certain MAbs or the presence of any free biotin.

The competition assays revealed complex, yet useful information concerning the relative binding sites of MAbs considered for detection studies. Although no two antibodies appeared to recognize the same or overlapping epitopes, results gave an indication of the proximity of MAb binding sites. Those MAbs showing the closest binding sites included antibodies 27.150.3, 43.11.5.1 and 40.18.7.1, all previously shown to be Rc reactive. The double MAb sandwich ELISAs developed, employed the most suitable MAb combinations for the detection of specific endotoxins.

The O-antigen polysaccharide chains, formed by the polymerization of repeating units, will perhaps represent the most suitable LPS component detectable in such an assay because of the availability and accessibility of multiple epitopes. Indeed, this was reflected in both the good sensitivity (0.01 ng m^{-1} purified *E. coli* 018 LPS) and ability to use the same MAb (30.4.2.8) as both the primary capture antibody and the secondary detector probe. The ability of MAb 30.4.2.8 to detect other non-018 LPSs at high concentrations only, reflects the antibody's low affinity for other LPS components as described earlier. Those assays developed for the detection of *E. coli* core LPS proved more sensitive for purified R-LPS than equivalent S-LPS. These results perhaps reflect both the accessibility of relevant epitopes and the binding of most MAbs to unsubstituted R-LPS only. The two most cross-reactive MAbs, 27.150.3 and 43.11.5.1 were capable of detecting *E. coli* core types R1-R4 at between 0.1 and 10 ng ml^{-1} purified LPS. Poor sensitivity for R3 appears to relate to the weak affinity of MAb 43.11.5.1 for this core type. The detection of individual *E. coli* core types was afforded by MAbs reacting almost exclusively (43.3.4.8) or preferentially (27.193.3) against R1 and R3 core types respectively.

In addition to developing double MAb sandwich ELISAs, those based on the use of polymyxin B-MAb combinations were also attempted. Microtitre plates were coated with polymyxin B, a polypeptide antibiotic with strong affinity for lipid A (Morrison & Jacobs, 1976). *E. coli* LPS bound to polymyxin was detected by single labelled MAbs. Although the sensitivity was generally comparable to most sandwich ELISAs, preliminary results gave poor reproducibility and high backgrounds, thought to reflect non-specific binding of MAbs to the polymyxin. Offering the advantages of theoretically capturing all LPS molecules retaining the lipid A domain, and requiring only a single labelled antibody for the detection of specific LPS, the technique may warrant future consideration. Indeed, it has been applied successfully for the detection of *H. influenzae* LOS (Mertsola *et al*, 1989; 1990). The development of alternative immunoassay may also be of benefit. Limet *et al* (1988) reported an assay based on latex agglutination for the detection of *B. abortus* LPS; Luk & Lindberg (1991) employed an immunomagnetic technique to detect specific *Salmonella* O-antigen LPSs, and Gagliardi *et al* (1986) developed a biotin-avidin amplified inhibition assay for the detection of lipid A.

The double MAb sandwich ELISAs developed in this study detected a minimum concentration of 0.01 ng ml^{-1} purified LPS in solution, although were less sensitive against certain purified LPS samples. The clinical usefulness of endotoxin assays such as these will depend on their ability to detect the relatively low concentrations of endotoxin in blood of between 0.005 and 0.1 ng ml^{-1}, indicative of endotoxaemia (Fomsgaard, 1990; Bottoms *et al*, 1991). However, there are many problems associated with the quantification of LPS in blood because endotoxin or lipid A binds to several substances present in plasma or

serum: natural antibodies (Gagliardi *et al*, 1986), high and low density lipoproteins, complement, serum albumin and acute phase and some other proteins (Ulevitch & Tobias, 1988). Preliminary experiments involving measurement of serum spiked with purified LPS showed that serum definitely has a significant effect on the ability of the assay to detect *E. coli* LPS. Similar findings have been reported by Gagliardi *et al* (1986) and Mertsola *et al* (1989).

The detection of LPS in blood serum has been demonstrated following various methods of deproteinization which, in this study, were largely unsuccessful in producing the required sensitivity. The isolation of LPS from serum using a phenol extraction method appeared to offer greater promise than other methods attempted. Preliminary results however, were not reproducible and methods other than overnight dialysis to remove phenol, such as desalting columns, need to be considered for future studies. Further investigations will also need to assess the ability of assay systems employing antibodies to detect LPS *in vivo*. Sensitivity will again be influenced by the physio-chemical presentation of LPS, which will affect both availability and accessibility of specific epitopes. Bacterial death and subsequent cell lysis may result in liberation of outer membrane fragments of different sizes, as well as free LPS molecules or their aggregates (Munford *et al*, 1984). The presentation of host LPS as monovalent fragments for example, would render a double sandwich MAb assay useless. Speculation also exists about the most suitable test material for the detection of LPS in blood. LPS adheres to cellular as well as humoral components in blood, therefore cell-bound endotoxin will be missed when serum or plasma is chosen as a sample (Tamura *et al*, 1991).

The clinical utility of the immunoassays described herein is clearly dependent on further studies which improve assay sensitivity in whole blood or serum/plasma. However, these detection systems are potentially useful tools for measurement of endotoxins with great specificity.

REFERENCES

REFERENCES

AISEN, P. & LEIBMAN, A. (1972) Lactoferrin and transferrin: a comparative study. Biochim. Biophys. Acta 257, 314-323.

ALCOCK, S.R. & LEDINGHAM, I.Mc.A. (1988) Selective decontamination of the digestive tract and prevention of infection in intensive care units. J. Antimicrob. Chemother. 22, 97-104.

ANDERSON, M.S., BULAWA, C.E. & RAETZ, C.R.H. (1985) The biosynthesis of Gram-negative endotoxin: formation of lipid A precursors from UDP-GlcNAc in extracts of *Escherichia coli*. J. Biol. Chem. 260, 15536-15541.

APPELMELK, B., GRUTEKE, P., VERWEIJ-VAN VUGHT, M., MAASKANT, J., THIJS, B. & MACLAREN, D. (1987) Measurement in human sera of antibodies to lipopolysaccharides of *Escherichia coli* J5. Microb. Pathogen. 2, 391-393.

APPELMELK, B.J., RAPSON, N.T., VERWEIJ-VAN VUGHT, A.M.J.J., MAASKANT, J.J., HEKKER, T.A.M., PERRBOOMS, P.G.H., MACLAREN, D.M. & THIJS, L.G. (1986a) Heterogeneity of *Escherichia coli* J5 vaccines. Lancet II, 1273-1274.

APPELMELK, B.J., VERWEIJ-VAN VUGHT, A.M.J.J., MACLAREN, D.M. & THIJS, L.S. (1985) An enzyme linked immunosorbent assay (ELISA) for the measurement of antibodies to different parts of the Gram-negative lipopolysaccharide core region. J. Immunol. Methods 82, 199-207.

APPELMELK, B.J., VUGHT, V.-V., MAASKANT, J.J., SCHOUTEN, W.F., THIJS, L.G. & MACLAREN, D.M. (1986b) Use of mucin and hemoglobin in experimental murine Gram-negative bacteremia enhances the immunoprotective action of antibodies reactive with the lipopolysaccharide core region. Antonie van Leeuwenhoek 52, 537-542.

ARAJ, G.F. & DAS CHUGH, T. (1987) Detection of *Salmonella* spp. in clinical specimens by capture enzyme-linked immunosorbent assay. J. Clin. Microbiol. 25, 2150-2153.

ARTENSTEIN, A.W. & CROSS, A.S. (1989) Inhibition of endotoxin reactivity by aminoglycosides. Correspondence. J. Antimicrob. Chemother. 24, p826-828.

ATKINSON, B.A. & AMARAL, L. (1982) Sublethal concentrations of antibiotics, effects on bacteria and the immune system. Crit. Rev. Microbiol. 9, 101-138.

AYDINTUG, M.K., INZANA, T.J., LETONJA, T., DAVIS, W.C. & CORBEIL, L.B. (1989) Cross-reactivity of monoclonal antibodies to *Escherichia coli* J5 with heterologous Gram-negative bacteria and extracted lipopolysaccharides. J. Infect. Dis. 160, 846-857.

BALDWIN, G., ALPERT, G., CAPUTO, G.L., BASKIN, M., PARSONNET, J., GILLIS, Z.A., THOMPSON, C., SIBER, G.R. & FLEISHER, G.R. (1991) Effect of polymyxin B on experimental shock from *Meningococcal* and *Escherichia coli* Endotoxins. J. Infect. Dis. 164, 542-549.

BARCLAY, G.R. (1990) Antibodies to endotoxin in health and disease. Rev. Med. Micro. 1, 133-142.

BARCLAY, G.R. & SCOTT, B.B. (1987) Serological relationships between *E. coli* and *Salmonella* smooth- and rough- mutant lipopolysaccharides as revealed by ELISA for human IgG antiendotoxin antibodies. Infect. Immun. 55, 2706-2714.

BARCLAY, G.R., SCOTT, B.B., WRIGHT, I.H., ROGERS, P.N., SMITH, D.G.E. & POXTON, I.R. (1989) Changes in anti-endotoxin-IgG antibody and endotoxaemia in three cases of Gram-negative septic shock. Circ. Shock 29, 93-106.

BATE, C.A.W., TAVERNE, J. & PLAYFAIR, J.H.L. (1988) Malarial parasites induce TNF production by macrophages. Immunology 64, 227-231.

BAUMGARTNER, J.-D. & GLAUSER, M.P. (1987) Controversies in the use of passive immunotherapy for bacterial infections in the critically ill patient. Rev. Infect. Dis. 9, 194-205.

BAUMGARTNER J.-D., GLAUSER, M.P., McCUTCHAN, J.A., ZIEGLER, E.J., VAN MELLE, G., KLAUBER, M.R., VOGT, M., MUEHLEN, E., LUETHY, R., CHIOLERO, R. & GEROULANOS, S. (1985) Prevention of Gram-negative shock and death in surgical patients by antibody to endotoxin core glycolipid. Lancet II, 59-63.

BAUMGARTNER, J.D., HEUMANN, D., CALANDRA, T. & GLAUSER, M.P. (1991) Antibodies to lipopolysaccharides after immunization of humans with the rough mutant *Escherichia coli* J5. J. Infect. Dis. 163, 769-772.

BAUMGARTNER, J.D., O'BRIEN, T.X., KIRKLAND, T.N., GLAUSER, M.P. & ZIEGLER, E.J. (1987) Demonstration of cross-reactive antibodies to smooth Gram-negative bacteria in antiserum to *Escherichia coli* J5 antiserum. J. Infect. Dis. 156, 136-143.

BAYSTON, K.F. & COHEN, J. (1990) Bacterial endotoxin and current concepts in the diagnosis and treatment of endotoxaemia. J. Med. Microbiol. 31, 73-83.

BERNARD, G.R., GROSSMAN, J.E., GAMPBELL, G.D. (1989) Multicenter trial of a monoclonal antiendotoxin antibody (Xomen-E5) in Gram-negative sepsis. Chest, 96, 137S. Abstract.

BERRY, A., DE VAULT, J.D., ROYCHOUDHURY, S., ZIELINSKI, N.A., MAY, T.B., ROTHMEL, R.K., FIALHO, A.M., HUSSEIN, M., KRYLOV, V. & CHAKRABARTY, A.M. (1988) *Pseudomonas aeruginosa* infection in cystic fibrosis: molecular approaches to a medical problem. Chimicaoggi, September, 13-19.

BETLEY, M.J., MILLER, V.L. & MEKALANOS, J.J. (1986) Genetics of bacterial enterotoxins. Annu. Rev. Microbiol. 40, 577-605.

BEUTLER, B., MILSARK, I.W. & CERAMI, A.C. (1985) Passive immunization against cachectin/tumor necrosis factor protects mice from lethal effects of endotoxin. Science 229, 869-871.

BHRIAIN, N.NI., DORMAN, C.J. & HIGGINS, C.F. (1989) An overlap between osmotic and anaerobic stress responses: a potential role for DNA supercoiling in the coordinate regulation of gene expression. Mol. Microbiol. 3, 933-942.

BOGARD, W.C., DUNN, D.L., ABERNETHY, K., KILGARRIFF, C. & KUNG, P.C. (1987) Isolation and characterization of murine monoclonal antibodies specific for Gram-negative bacterial lipopolysaccharide: association of cross-genus reactivity with lipid A specificity. Infect. Immun. 55, 899-908.

BOGARD, W.C., Jr. & SIEGEL, S.A. (1991) The human monoclonal antibody HA-1A: Studies on the epitope location within the endotoxin molecule and epitopic exposure on the surface of viable Gram-negative bacteria. Abstract 332. Circ. Shock 34, p119.

BOIVIN, A. & MESROBEANU, L. (1933) Rùcherches sur les antigñnes somatiques et sur les endotoxines des bactùries. IV sur L'action anti-endotoxique de l'anticorps. Rev. Immunol. Ther. Antimicrob. 4, 40-56.

BONE, R.C., FISHER, C.J., Jr., CLEMMER, T.P., STOTMAN, G.J., METZ, C.A. & BALK, R.A. (1989) Sepsis syndrome: a valid clinical entity. Crit. Care Med. 17, 389-393.

BOTTOMS, G.D., GIMARS, S. & PFEIFER, C. (1991) Plasma concentrations of endotoxin following jugular or portal injections of endotoxin and following gastrointestinal ischemia due to hemorrhage. Circ. Shock 33, 1-6.

BOURRET, R.B., HESS, J.F., BORKOVICH, K.A., PAKULA, A.A. & SIMON, M.I. (1989) Protein phosphorylation in chemotaxis and two component regulatory systems of bacteria. J. Biol. Chem. 264, 7085-7088.

BOYCE, J.R. & MILLER, R.V. (1982) Selection of non-mucoid derivatives of mucoid *Pseudomonas aeruginosa* is strongly influenced by the level of iron in the culture medium. Infect. Immun. 37, 695-701.

BOYE, E., STEEN, H.B. & SKARSTAD, K. (1983) Flow cytometry of bacteria: a promising tool in experimental and clinical microbiology. J. Gen. Microbiol. 129, 973-980.

BRADE, H., BRADE, L., KACA, W., TACKEN, A. & RIETSCHEL, E.T. (1986) Structural analysis of the inner core region of Enterobacterial lipopolysaccharides. EOS-Riv. Immunol. Immunofarm. 6, (Suppl.), 26-28.

BRADE, H., BRADE, L. & RIETSCHEL, E.T. (1988) Structure-activity relationships of bacterial lipopolysaccharides (endotoxins). Current and future aspects. Zbl. Bakt. Hyg. 268, 151-179.

BRADE, H., MOLL, H. & RIETSCHEL, E.T. (1985). Structural investigations on the inner core of lipopolysaccharide from *Salmonella minnesota* rough mutants. Biomed. Mass. Spectrom. 12, 602-609.

BRADE, H. & RIETSCHEL, E.T. (1984) Alpha-2-4 interlinked 3-deoxy-D-manno-octulosonic acid disaccharide. A common constituent of enterobacterial lipopolysaccharides. Eur. J. Biochem. 145, 231-236.

BRADE, L. & BRADE, H. (1985) A 28,000-dalton protein of normal mouse serum binds specifically to the inner core region of bacterial lipopolysaccharide. Infect. Immun. 50, 687-694.

BRADE, L., BRANDENBURG, K., KUHN, H.-M., KUSUMOTO, S., MACHER, I., RIETSCHEL, E.T. & BRADE, H. (1987a) The immunogenicity and antigenicity of lipid A are influenced by its physiochemical state and environment. Infect. Immun. 55, 2636-2644.

BRADE, L., KOSMA, P., APPELMELK, B.J., PAULSEN, H. & BRADE, H. (1987b) Use of synthetic antigens to determine the epitope specificities of monoclonal antibodies against the 3-deoxy-D-manno-octulosonate region of bacterial lipopolysaccharide. Infect. Immun. 55, 462-466.

BRAHMBHATT, H.N., WYK, P., QUIGLEY, N.B. & REEVES, P.R. (1988) Complete physical map of the *rfb* gene cluster encoding biosynthetic enzymes for the O-antigen of *Salmonella typhimurium* LT2. J. Bacteriol. 170, 98-102.

BRANDENBURG, K. & SEYDEL, U. (1984) Physical aspects of structure and function of membranes made from lipopolysaccharides and free lipid A. Biochim. Biophys. Acta 775, 225-238.

BRAQUET, P., TOUQUI, L., SHEN, T.Y. & VARGAFTIG, B.B. (1987) Perspectives in platelet-activating factor research. Pharmacol. Rev. 39, 97-145.

BRASS, J.M. (1986) The cell envelope of Gram-negative bacteria: new aspects of its function in transport and chemotaxis. Curr. Top. Microbiol. Immunol. 129, 1-92.

BRAUDE, A.I. & DOUGLAS, H. (1972) Passive immunization against the local Shwartzman reaction. J. Immunol. 108, 505-512.

BRAUDE, A.I., DOUGLAS, H. & DAVIS, C.E. (1973) Treatment and prevention of intracellular coagulation with antiserum to endotoxin. J. Infect. Dis. 128, (Suppl.): S157-S164.

BRILL, N.E. & LIBMAN, E. (1899) Pyocyaneus bacillaemia. A critical review of the recorded cases, with the report of a case secondary to staphylococcaemia. Am. J. Med. Sci. 118, 153-162.

BROUGHTON, A., ANDERSON, I.R.M. & BOWDEN, C.H. (1968) Magnesium-deficiency syndrome in burns. Lancet II, 1156-1158.

BROWN, M.R.W., ANWAR, H. & LAMBERT, P.A. (1984) Evidence that mucoid *Pseudomonas aeruginosa* in the cystic fibrosis lung grows under iron-restricted conditions. Fed. Eur. Microbiol. Soc. Microbiol. Lett. 21, 113-117.

BROWN, M.R.W. & WILLIAMS, P. (1985) The influence of environment on envelope properties affecting survival of bacteria in infections. Annu. Rev. Microbiol. 39, 527-556.

BROZEK, K.A., HOSAKA, K., ROBERTSON, A.D. & RAETZ, C.R.H. (1989) Biosynthesis of lipopolysaccharide in *Escherichia coli*. J. Biol. Chem. 264, 6956-6966.

BRUUN, B., SLOTH, K., BENTZON, M.W. & FREDERIKSEN, W.A. (1982) A study of bacteraemias in Denmark from 1977-1978. Acta Pathol. Microbiol. Scand. B. 90, 309-317.

BRYAN, C.S., REYNOLDS, K.L. & BRENNER, E.R. (1983) Analysis of 1,186 episodes of Gram-negative bacteremia in non-university hospitals: the effects of antimicrobial therapy. Rev. Infect. Dis. 5, 629-638.

BRYANT, R.E., HOOD, C.E. & KOENIG, M.G. (1971) Factors affecting mortality of Gram-negative rod bacteremia. Arch. Intern. Med. 127, 120-128.

BULLEN, J.J. (1981) The significance of iron in infection. Rev. Infect. Dis. 3, 1127-1138.

CADIEUX, J.E., KUZIO, J., MILAZZO, F.H. & KROPINSKI, A.M. (1983) Spontaneous release of lipopolysaccharide by *Pseudomonas aeruginosa*. J. Bacteriol. 155, 817-825.

CADY, A.B., KOTANI, S., SHIBA, T., KUSUMOTO, S. & KRUEGER, J.M. (1989) Somnogenic activities of synthetic lipid A. Infect. Immun. 57, 396-403.

CALANDRA, T., GLAUSER, M.P., SCHELLEKENS, J. & VERHOEF, J. & the Swiss-Dutsch J5 Immunoglobulin Study Group (1988) Treatment of Gram-negative septic shock with human IgG antibody to *Escherichia coli* J5: a prospective, double-blind, randomized trial. J. Infect. Dis. 158, 312-319.

CAVAILLON J.-M., FITTING, C., HAEFFNER-CAVAILLON, N., KIRSCH, S.J. & WARREN, H.S. (1990) Cytokine response by monocytes and macrophages to free and lipoprotein-bound lipoplysaccharide. Infect. Immun. 58, 2375-2382.

CERRA, F.B. (1987) Hypermetabolism, organ failure and metabolic support. Surgery 101, 1-14.

CHEASTY, T., GROSS, R.J. & ROWE, B. (1977) Incidence of K1 antigen in *Escherichia coli* isolated from blood and cerebrospinal fluid of patients in the United Kingdom. J. Clin. Pathol. 30, 945-947.

CHEDID, L., PARANT, M., PARANT, F. & BOYER, F. (1968) A proposed mechanism for natural immunity to enterobacterial pathogens. J. Immunol. 100, 292-301.

CHESTER, I.R. & MEADOW, P.M. (1975) Heterogeneity of the lipopolysaccharide from *Pseudomonas aeruginosa*. Eur. J. Biochem. 58, 273-282.

CHIA, J.K.S., POLLACK, M., GUELDE, G., KOLES, N.L., MILLER, M. & EVANS, M. (1989) Lipopolysaccharide (LPS)-reactive monoclonal antibodies fail to inhibit LPS-induced tumor necrosis factor secretion by mouse-derived macrophages. J. Infect. Dis. 159, 872-880.

CHONG, K.T. & HUSTON, M. (1987) Implications of endotoxin contamination in the evaluation of antibodies to lipopolysaccharides in a murine model of Gram-negative sepsis. J. Infect. Dis. 156, 713-719.

CIANCIO, M.J., HUNT, J., JONES, S.B. & FILKINS, J.P. (1991) Comparative and interactive *in vivo* effects of tumor necrosis factor α and endotoxin. Circ. Shock 33, 108-120.

CIURANA, B. & TOMAS, J.M. (1987) Role of lipopolysaccharide and complement in susceptibility of *Klebsiella pneumoniae* to non-immune serum. Infect. Immun. 55, 2741-2746.

CLARK, V.L. (1990) Environmental modulation of gene expression in Gram-negative pathogens. In Molecular Basis of Bacterial Pathogenesis, pp 111-135. Edited by B.H. Iglewski & V.L. Clark. Academic Press, San Diego.

CLEGG, S. & GERLACH, G.F. (1987) Enterobacterial fimbriae. J. Bacteriol. 169, 934-938.

COHEN, J. (1986) Anti-endotoxin immunotherapy in septic shock J. Antimicrob. Chemother. 18, 436-439.

COHEN, J. (1988) Intravenous immunoglobulin (IVIG) for Gram-negative infection: a critical review. J. Hosp. Infect. 12, (Suppl. D), 47-54.

COHEN, J. (1989) Endotoxin-significance, detection and treatment. In Recent Advances in Infection, pp 131-140. Edited by D.S. Reeves & A.M. Geddes. Churchill Livingstone, Edinburgh.

COHEN, J. & McCONNELL, J.S. (1984) Observations on the measurement and evaluation of endotoxemia by a quantitative Limulus lysate microassay. J. Infect. Dis. 150, 916-924.

COHEN, J. & McCONNELL, J.S. (1985) Antibiotic-induced endotoxin release. Lancet II, 1069.

COHEN, J. & McCONNELL, J.S. (1986) Release of endotoxin from bacteria exposed to ciprofloxacin and its prevention with polymyxin B. Eur. J. Clin. Microbiol. 5, 13-17.

COLEMAN, J. & RAETZ, C.R.H. (1988) First committed step of lipid A biosynthesis in *Escherichia coli* sequence of the *lpxA* gene. J. Bacteriol. 170, 1268-1274.

COLLINS, H.H., CROSS, A.S., DOBEK, A., OPAL, S.M., McCLAIN, J.B. & SADOFF, J.C. (1989) Oral ciprofloxacin and a monoclonal antibody to lipopolysaccharide protect leukopenic rats from lethal infection with *Pseudomonas aeruginosa*. J. Infect. Dis. 159, 1073-1082.

COLLINS, M.S., TSAY, G.C., HECTOR, R.F., ROBY, R.E. & DORSEY, J.H. (1986) Immunoglobulin G: potentiation of tobramycin and azlocillin in the treatment of *Pseudomonas aeruginosa* sepsis in neutropenic mice and neutralization of exotoxin A *in vivo*. Rev. Infect. Dis. 8, (Suppl. 4), S420-S425.

COLWELL, D.E., MICHALEK, S.M., BRILES, D.E., JIRILLO, E. & McGHEE, J.R. (1984) Monoclonal antibodies to *Salmonella* lipopolysaccharide: anti-O-polysaccharide antibodies protect C3H mice against challenge with virulent *Salmonella typhimurium*. J. Immunol. 133, 950-957.

COOPER, N.R. & MORRISON, D.C. (1978) Binding and activation of the first component of human complement by the lipid A region of lipopolysaccharide. J. Immunol. 120, 1862-1868.

CORNELISSEN, J.J., VAN KESSEL, C.P.M., BROUWER, E., KRAAIJEVELD, C.A. & VERHOEF, J. (1991) Inhibition by lipid A-specific monoclonal antibodies of priming of human polymorphonuclear leucocytes by endotoxin. J. Med. Microbiol. 34, 233-238.

COSTERTON, J.W. (1984) The etiology and persistence of cryptic bacterial infections: a hypothesis. Rev. Infect. Dis. 6, (Suppl. 3), S608-S616.

COSTERTON, J.W., INGRAM, J.M. & CHENG, K.-J. (1974) Structure and function of the cell envelope of Gram-negative bacteria. Bact. Rev. 38, 87-110.

COUGHLIN, R.T., ZISSOMOS, E.M., KILGARRIFF, C., QUINN, A., OBERHOLTZER, C. & BOGARD, W.C. (1986) Lipid A specific antibodies protect against experimental sepsis. Riv. Immunol. Immunofarmacol. 6, 260.

CRAIG, W.A., TURNER, J.H. & KUNIN, C.M. (1974) Prevention of the generalized Shwartzman reaction and endotoxin lethality by polymymix B localized in tissues. Infect. Immun. 10, 287-289.

CROSS, A.S. (1990) The biological significance of bacterial encapsulation. Curr. Top. Microbiol. Immunol. 150, 87-95.

CROSS, A.S., GEMSKI, P., SADOFF, J.C., ØRSKOV, F. & ØRSKOV, I. (1984) The importance of the K1 capsule in invasive infections caused by *Escherichia coli*. J. Infect. Dis. 149, 184-193.

CROSS, A.S., KIM, K.S., WRIGHT, D.C., SADOFF, J.C. & GEMSKI, P. (1986) Role of lipopolysaccharide and capsule in serum resistance of bacteraemic strains of *Escherichia coli*. J. Infect. Dis. 154, 497-503.

CROSS, A.S., SIDBERRY, H. & SADOFF, J.C. (1989) The human antibody response during natural bacteremic infection with Gram-negative bacilli against lipopolysaccharide core determinants. J. Infect. Dis. 160, 225-236.

CRYZ, S.J., Jr., CROSS, A.S., SADOFF, J.C. & FÜRER, E. (1990) Synthesis and characterization of *Escherichia coli* 018 O-polysaccharide conjugate vaccines. Infect. Immun. 58, 373-377.

CRYZ, S.J., Jr., CROSS, A.S., SADOFF, J.C. & FÜRER, E. (1991) Safety and immunogenicity of *Escherichia coli* 018 O-specific polysaccharide (O-PS)-toxin A and O-PS-cholera toxin. J. Infect. Dis. 163, 1040-1045.

CRYZ, S.J., Jr., FÜRER, E. & GERMANIER, R. (1984) Protection against fatal *Pseudomonas aeruginosa* burn wound sepsis by immunization with lipopolysaccharide and high molecular weight polysaccharide. Infect. Immun. 43, 795-799.

CRYZ, S.J., Jr., MEADOW, P.M., FÜRER, E. & GERMANIER, R. (1985) Protection against fatal *Pseudomonas aeruginosa* sepsis by immunization with smooth and rough lipopolysaccharides. Eur. J. Clin. Microbiol. 4, 180-185.

CRYZ, S.J., Jr., SADOFF, J.C., CROSS, A.S. & FÜRER, E. (1989) Safety and immunogenicity of a polyvalent *Pseudomonas aeruginosa* O-polysaccharide-toxin A vaccine in humans. Antibiot. Chemother. 42, 177-183.

CUEVAS, P. & FINE, J. (1972) Role of intestinal endotoxin in death from peritonitis. Surg. Gynecol. Obst. 134, 953-957.

DANNER, R.L., ELIN, R.J., HOSSEINI, J.M., WESKY, R.A., REILLY, J.M. & PARRILLO, J.E. (1991) Endotoxemia in human septic shock. Chest 99, 169-175.

DANNER, R.L., JOINER, K.A. & PARRILLO, J.E. (1987) Inhibition of endotoxin-induced priming of human neutrophils by lipid X and 3-aza-lipid X. J. Clin. Invest. 80, 605-612.

DARVEAU, P.P. & CUNNINGHAM, M.D. (1990) Influence of sub-inhibitory concentrations of cephalosporins on the serum sensitivity of *Pseudomonas aeruginosa*. J. Infect. Dis. 162, 914-921.

DAVEY, P., GOZZARD, D., GOODALL, M. & LEYLAND, M.J. (1985) Hypomagnesaemia: an underdiagnosed interaction between gentamicin and cytotoxic chemotherapy for acute non-lymphoblastic leukaemia. J. Antimicrob. Chemother. 15, 623-628.

DAVIS, C.E., ZIEGLER, E.J. & ARNOLD, K.F. (1978) Neutralization of meningococcal endotoxin by antibody to core glycolipid. J. Exp. Med. 147, 1007-1017.

DAY, D.F. & MARCEAU-DAY, M.L. (1982) Lipopolysaccharide variability in *Pseudomonas aeruginosa*. Curr. Microbiol. 7, 93-98.

DEITCH, E.A., BRIDGES, R.Mc.I., DOBKE, M. & McDONALD, J.C. (1987) Burn wound sepsis may be promoted by a failure of local antibacterial host defenses. Ann. Surg. 206, 340-348.

DE JONGH-LEUVENINK, J., BOUTER, A.S., MARCELIS, J.H., SCHELLEKENS, J. & VERHOEF, J. (1986) Cross-reactivity of monoclonal antibodies against lipopolysaccharides of Gram-negative bacteria. Eur. J. Clin. Microbiol. 5, 148-151.

DEMARCO DE HORMAECHE, R., JESSOP, H. & SENIOR, K. (1988) Gonococcal variants selected by growth *in vivo* or *in vitro* have antigenically different LPS. Microb. Pathogen. 4, 289-297.

DeMARIA, A., Jr., JOHNS, M.A., BERBERICH, H. & McCABE, W.R. (1988) Immunization with rough mutants of *Salmonella minnesota*: initial studies in human subjects. J. Infect. Dis. 158, 301-311.

DERETIC, V., TOMASEK, P., DARZINS, A. & CHAKRABARTY, A.M. (1986) Gene amplification induces mucoid phenotype in rec-2 *Pseudomonas aeruginosa* exposed to kanomycin. J. Bacteriol. 165, 510-516.

VAN DEVENTER, S.J.H., BULLER, H.R., TEN CATE, J.W., STURK, A. & PAUW, W. (1988a) Endotoxaemia: an early predictor of septicaemia in febrile patients. Lancet I, 605-609.

VAN DEVENTER, S.J.H., TEN CATE, J.W. & TYTGAT, G.N.J. (1988b) Intestinal endotoxaemia: clinical significance. Gastroenterology 94, 825-831.

DEVINE, D.A., ROBERTS, A.P. & ROWE, B. (1990) Simple technique for detecting K1 antigen of *Escherichia coli*. J. Clin. Pathol. 43, 76-78.

DEWITT, C.W. & ROWE, J.A. (1961) Sialic acids (N, 7-O-diacetylneuraminic acid and N-acetylneuraminic acid) in *Escherichia coli*. 1. Isolation and identification. J. Bacteriol. 82, 838-848.

DICK, J.D., SHULL, V., KARP, J.E. & VALENTINE, J. (1988) Bacterial and host factors affecting *Pseudomonas aeruginosa* colonization versus bacteraemia in granulocytopenic patients. Eur. J. Cancer Clin. Oncol. 24, (Suppl.), S47-S54.

DINARELLO, C.A. (1984) Interleukin-1. Rev. Infect. Dis. 6, 51-95.

DINARELLO, C.A. (1989) Interleukin 1 and its biologically related cytokines. Adv. Immunol. 44, 153-205.

DIPIRO, J.T. (1990) Pathophysiology and treatment of Gram-negative sepsis. Am. J. Hosp. Pharm. 47, S6-S10.

DODDS, K.L., PERRY, M.B. & McDONALD, I.J. (1987) Alteration in LPS produced by chemostat grown *Escherichia coli* O157:H7 as a function of growth rate and growth limiting nutrient. Can. J. Microbiol. 33, 452-458.

DOETSCH, R.N. & SJOBLAD (1980) Flagellar structure and function in eubacteria. Ann. Rev. Microbiol. 34, 69-108.

DORMAN, C.J. (1991) DNA supercoiling and environmental regulation of gene expression in pathogenic bacteria. Infect. Immun. 59, 745-749.

DORMAN, C.J., BARR, G.C., BHRIAIN, N.NI. & HIGGENS, C.F. (1988) DNA supercoiling and the anaerobic and growth phase regulation of tonB gene expression. J. Bact. 170, 2816-2826.

DREWRY, D.T., LOMAX, J.A., GRAY, G.W. & WILKINSON, S.G. (1973) Studies of lipid A fractions from the lipopolysaccharides of *Pseudomonas aeruginosa* and *Pseudomonas alcaligenes*. Biochem. J. 133, 563-572.

DUDLEY, M.N. (1990) Overview of gram-negative sepsis. Am. J. Hosp. Pharm. 47, (Suppl. 3), S3-S6.

DUNN, D.L., BOGARD, W.C. & CERRA, F.B. (1985) Efficacy of type-specific and cross-reactive murine monoclonal antibodies directed against endotoxin during experimental sepsis. Surgery 98, 283-290.

DUNN, D.L., EWALD, D.C., CHANCLAN, N. & CERRA, F.B. (1986) Immunotherapy of Gram-negative bacterial sepsis. A single murine monoclonal antibody provides cross-genera protection. Arch. Surg. 121, 58-62.

DUNN, D.L., PRIEST, B.P. & CONDIE, R.M. (1988) Protective capacity of polyclonal and monoclonal antibodies directed against endotoxin during experimental sepsis. Arch. Surg. 123, 1389-1393.

DUSWALD K.H., MÜLLER, K., SEIFERT, J. & RING, J. (1980) Effectiveness of intravenous gammaglobulin against bacterial infections in surgical patients. Münch. Med. Wschr. 122, 832-836.

EASMON, C.S.F. (1990) Pathogenesis of septicaemia. J. Antimicrob. Chemother. 25, Suppl. C, 9-16.

EDWARDS, M.S., KASPER, D.L., JENNINGS, H.J., BAKER, C.J. & NICHOLSON-WELLER, A. (1982) Capsular sialic acid prevents activation of the alternative pathway by type III, group B streptococci. J. Immunol. 128, 1278-1283.

ELKINS, K. & METCALF, E.S. (1985) Binding activity of a murine anti-lipid A antibody. Infect. Immun. 48, 597-600.

ELLISON, R.T., 111, GIEHL, T.J. & LAFORCE, F.M. (1988) Damage of the outer membrane of enteric Gram-negative bacteria by lactoferrin and transferrin. Infect. Immun. 56, 2774-2781.

ELLISON, R.T., 111, LAFORCE, F.M., GIEHL, T.J., BOOSE, D.S. & DUNN, B.E. (1990) Lactoferrin and transferrin damage of the Gram-negative outer membrane is modulated by Ca^{2+} and Mg^{2+}. J. Gen. Micro. 136, 1437-1446.

ENG, R.H.K., BISHBURG, E., SMITH, S.M., GELLER, H. & KAPILA, R. (1987) Bacteraemia and fungaemia in patients with the acquired immune deficiency syndrome. Am. J. Clin. Pathol. 86, 105-107.

ESKENAZY, M., KONSTANTINOV, G., IVANOVA, R. & STRAHILOV, D. (1977) Detection by immunofluorescence of common antigenic determinants in unrelated Gram-negative bacteria and their lipopolysaccharides. J. Infect. Dis. 135, 965-969.

ESSIG, P., MARTIN, H.H. & GMEINER, J. (1982) Murein and lipopolysaccharide biosynthesis in synchronized cells of *Escherichia coli* K12 and the effect of penicillin G, mecillinam, and nalidixic acid. Arch. Microbiol. 132, 245-250.

EVANS, M.E., POLLACK, M., HARDEGEN, N.J., KOLES, N.L., GUELDE, G. & CHIA, J.K.S. (1990) Fluorescence-activated cell sorter analysis of binding by lipopolysaccharide-specific monoclonal antibodies to Gram-negative bacteria. J. Infect. Dis. 162, 148-155.

EXLEY, A.R., COHEN, J., BUURMAN, W., OWEN, R., HANSON, G., LUMLEY, J., AULAKH, J.M., BODMER, M., RIDDELL, A., STEPHENS, S. & PERRY, M. (1990) Monoclonal antibody to TNF in severe septic shock. Lancet 335, 1275-1277.

EYKYN, S.J., GRANSDEN, W.R. & PHILLIPS, I. (1990) The causative organisms of septicaemia and their epidemiology. J. Antimicrob. Chemother. 25, (Suppl. C), 41-58.

FADEN, A.I. & HOLADAY, J.W. (1980) Experimental endotoxin shock: the pathophysiologic function of endorphins and treatment with opiate antagonists. J. Infect. Dis. 142, 229-238.

FALKOW, S. (1990) The "Zen" of bacterial pathogenicity. In Molecular Basis of Bacterial Pathogenesis pp 3-9. Edited by B.H. Iglewski & V.L. Clark. Academic Press Inc., San Diego.

FEIGE, U., JANN, B., JANN, K., SCHMIDT, G. & STIRM, S. (1977) On the primary structure of the R4 cell wall lipopolysaccharide core. Biochem. Biophys. Res. Commun. 79, 88-95.

FENWICK, B.W., CULLOR, J.S., OSBURN, B.I. & OLANDER, H.J. (1986) Mechanisms involved in protection provided by immunization against core lipopolysaccharides of *Escherichia coli* J5 from lethal *Haemophilus pleuropneumonia* infections in swine. Infect. Immun. 53, 298-304.

FINCH, J.E. & BROWN, M.R.W. (1975) The influence of nutrient limitation in a chemostat on the sensitivity of *Pseudomonas aeruginosa* to polymyxin and to EDTA. J. Antimicrob. Chemother. 1, 379-386.

FINLAY, B.B. & FALKOW, S. (1989) Common themes in microbial pathogenicity. Microbiol. Rev. 53, 210-230.

FINNE, J. (1982) Occurrence of unique polysialyl carbohydrate units in glycoprotein of developing brain. J. Biol. Chem. 257, 11966-11970.

FLOHÉ, L. & GIERTZ, H. (1987) Endotoxins, arachidonic acid, superoxide formation. Rev. Infect. Dis. 9, (Suppl.), S553-S561.

FLYNN, P.M., SHENEP, J.L., STOKES, D.C., FAIRCLOUGH, D. & HILDNER, W.K. (1987) Polymyxin B moderates acidosis and hypotension in established, experimental Gram-negative septicaemia. J. Infect. Dis. 156, 706-712.

FOMSGAARD, A. (1990) Antibodies to lipopolysaccharides: some diagnostic and protective aspects. APMIS 98 (Suppl. 18), 5-38.

FOMSGAARD, A., DINESEN, B. & BAEK, L. (1987) Anti-lipopolysaccharide antibodies measured by enzyme-immunoassay in danish blood donors. APMIS 95, 9-13.

FOMSGAARD, A., FREUDENBERG, M.A. & GALANOS, C. (1990) Modification of the silver staining technique to detect lipopolysaccharide in polyacrylamide gels. J. Clin Micro. 28, 2627-2631.

FOMSGAARD, A. & GALANOS, C. (1989) Protective properties of a human IgG preparation rich in antibodies to a wide spectrum of lipopolysaccharides. APMIS 97, 1114-1120.

FOMSGAARD, A., ZHANG, G.-H., SHAND, G.H., BENDTZEN, K. & BAEK, L. (1989) Immunochemical and biological reactivity of human anti-lipopolysaccharide IgG obtained by screening of blood donors. Scand. J. Immunol. 29, 309-316.

FONG, Y., TRACEY, K.J., MOLDAWER, L.L., HESSE, D.G., MANOGUE, K.B., KENNEY, J.S., LEE, A.T., KUO, G.C., ALLISON, A.C., LOWRY, S.F. & CERAMI, A. (1989) Antibodies to cachectin/tumor necrosis factor reduce interleukin 1β and interleukin 6 appearance during lethal bacteremia. J. Exp. Med. 170, 1627-1633.

FOWLER, A.A., HAMMAN, R.F., GOOD, J.T., BENSON, K.N., BAIRD, M., EBERLE, D.J., PETTY, T.L. & HYERS, T.M. (1983) Adult respiratory distress syndrome: risk with common predispositions. Ann. Intern. Med. 98, 593-597.

FOX, E.S., THOMAS, P. & BROITMAN, S.A. (1990) Hepatic mechanisms for clearance and detoxification of bacterial endotoxins. J. Nutri. Biochem. 1, 43-49.

FRAKER, D.L., STROVRAFF, M.C., MERINO, M.J., & NORTON, J.A. (1988) Tolerance to tumor necrosis factor in rats and the relationship to endotoxin tolerance and toxicity. J. Exp. Med. 168, 95-105.

FRANK, M.M., JOINER, K. & HAMMER, C. (1987) The function of antibody and complement in the lysis of bacteria. Rev. Infect. Dis. 9, (Suppl.), 537-545.

FREEMAN, R. & GOULD, F.K. (1985) Prevention of fever and Gram-negative infection after open heart surgery by antiendotoxin. Thorax 40, 846-848.

FRENCH, G.L., CHENG, A.F.B., DUTHIE, R. & COCKRAM, C.S. (1990) Septicaemia in Hong Kong. J. Antimicrob. Chemother. 25, Suppl. C, 115-125.

FRETER, R. & JONES, G.W. (1983) Models for studying the role of bacterial attachment in virulence and pathogenesis. Rev. Infect. Dis. 5, (Suppl. 4), 647-658.

FREUDENBURG, M.A., KEPPLER, D. & GALANOS, C. (1986) Requirement for lipopolysaccharide-responsive macrophages in galactosamine-induced sensitization to endotoxin. Infect. Immun. 51, 891-895.

FRIBERGER, P., SÖRSKOG, L., NILSSON, K. & KNÖS, M. (1987) The use of a quantitative assay in endotoxin testing. Progr. Clin. Biol. Res. 231, 149-169.

FULLER, N.A., WU WU M.-C., WILKINSON, R.G. & HEATH, E.C. (1973) The biosynthesis of cell wall lipopolysaccharide in *Escherichia coli*. VII. Characterization of heterogeneous core oligosaccharide structures. J. Biol. Chem. 248, 7938-7950.

FUNAHARA, Y. & NIKAIDO, H. (1980) Asymmetric localization of lipopolysaccharides on the outer membrane of *Salmonella typhimurium*. J. Bacteriol. 141, 1463-1465.

GAETA, G.B., PERNA, P., ADINOLFI, L.E., UTILI, R. & RUGGERIO, G. (1982) Endotoxaemia in a series of 104 patients with chronic liver diseases: prevalence and significance. Digestion 23, 239-244.

GAFFIN, S.L., BADSHA, N., BROCK-UTNE, J.E., VORSTER, B.J. & CONRADIE, J.D. (1982) An ELISA procedure for detecting human anti-endotoxin antibodies in serum. Ann. Clin. Biochem. 19, 191-194.

GAFFIN, S.L., BADSHA, N. & VORSTER, B.J. (1985) Properties of human anti-lipopolysaccharide gammaglobulin: specificity and protective effects. Vox. Sang. 48, 276-283.

GAGLIARDI, N.C., NOLAN, J.P., FEIND, D.M. & DeLISSIO, M. (1986) A rapid sensitive monoclonal assay for lipid A in solution. J. Immunol. Methods. 91, 243-247.

GALANOS, C., FREUDENBERG, M.A., NERKAR, F.J.D., VELEVA, K., BRADE, H. & STRITTMATTER, W. (1984) Immunogenic properties of lipid A. Rev. Infect. Dis. 6, 546-552.

GALANOS, C., HANSON-HAGGE, T., LEHMANN, V. & LÜDERITZ, O. (1985a) Comparison of the capacity of two lipid A precursor molecules to express the local Schwartzman phenomenon. Infect. Immun. 48, 355-358.

GALANOS, C. & LÜDERITZ, O. (1975) Electrodialysis of lipopolysaccharides and their conversion to uniform salt forms. Eur. J. Biochem. 54, 603-610.

GALANOS, C. & LÜDERITZ, O. (1984) Lipopolysaccharide: properties of an amphipathic molecule. In Handbook of Endotoxin. Vol. 1. Chemistry of endotoxin, 46-58. Edited by E.Th. Rietschel, Elsevier, Amsterdam.

GALANOS, C., LÜDERITZ, O., FREUDENBERG, M.A., BRADE, L., SCHADE, U., RIETSCHEL, E.T., KUSUMOTO, S. & SHIBA, T. (1986) Biological activity of synthetic heptaacyl lipid A representing a component of *Salmonella minnesota* R596 lipid A. Eur. J. Biochem. 160, 55-59.

GALANOS, C., LÜDERITZ, O., RIETSCHEL, E.TH., WESTPHAL, O., BRADE, H., BRADE, L., FREUDENBERG, M.A., SCHADE, U., IMOTU, M., YOSHIMURA, H., KUSUMOTO, S. & SHIBA, T. (1985b) Synthetic and natural *Escherichia coli* free lipid A express identical endotoxic activities. Eur. J. Biochem. 148, 1-5.

GALANOS, C., LÜDERITZ, O. & WESTPHAL, O. (1969) A new method for the extraction of R lipopolysaccharides. Eur. J. Biochem. 9, 245-249.

GALLOWAY, S.M. & RAETZ, C.R.H. (1990) A mutant of *Escherichia coli* defective in the first step of endotoxin biosynthesis. J. Biol. Chem. 265, 6394-6402.

GASTON, M.A., DUFF, P.S. & PITT, T.L. (1988) Lipopolysaccharide heterogeneity in strains of *Serratia marcescens* agglutinated by O14 antiserum. Curr. Microbiol. 17, 27-32.

GATELL, J.M., TRILLA, A., LATORRE, X., ALMELA, M., MENSA, J., MORENO, A., MIRO, J.M., MARTINEZ, J.A., JIMENEZ DE ANTO, M.T., SORIANO, E. & GARCIA SAN MIGUEL, J. (1988) Nosocomial bacteremia in a large Spanish teaching hospital: analysis of factors influencing prognosis. Rev. Infect. Dis. 10, 203-210.

GEDDES, A.M. (1988) Antibiotic therapy: a resume. Lancet I, 286-290.

GEMMELL, C.G. (1987) Changes in expression of bacterial surface antigens induced by antibiotics and their influence on host defences. Path. Biol. 35, 1377-1381.

GEMSKI, P., CROSS, A.S. & SADOFF, J.C. (1980) K1 antigen associated resistance to the bactericidal activity of serum. FEMS Microbiol. Lett. 9, 193-197.

GIGLIOTTI, F. & SHENEP, J.L. (1985) Failure of monoclonal antibodies to core glycolipid to bind intact smooth strains of *Escherichia coli*. J. Infect. Dis. 151, 1005-1011.

GILBERT, P. & BROWN, M.R.W. (1978) Influence of growth rate and nutrient limitation on the gross cellular composition of *Pseudomonas aeruginosa* and its resistance to 3- and 4-chlorophenol. J. Bacteriol. 133, 1066-1072.

GILLELAND, H.E., Jr. (1988) Adaptive altrations in the outer membrane of gram-negative bacteria during human infection. Can. J. Microbiol. 34, 499-502.

GLINZ, W., GROB, P.J., NYDEGGER, U.E., RICKLIN, T., STAMM, F., STOFFEL, D. & LASANCE, A. (1985) Polyvalent immunoglobulins for prophylaxis of bacterial infections in patients following multiple trauma. Intensive Care Med. 11, 288-294.

GOLDBLUM, S.E., COHEN, D.A. & MICHAEL, J. (1987) Interleukin 1 - induced depression of iron and zinc: role of granulocytes and lactoferrin. Am. J. Physiol. (Endocrin. Metab. 15), 252, E27-E32.

GOLDMAN, R.C., JOINER, K. & LEIVE, L. (1984) Serum-resistant mutants of *Escherichia coli* 0111 contain increased lipopolysaccharide, lack an O-antigen-containing capsule, and cover more of their lipid A core with O-antigen. J. Bacteriol. 159, 877-882.

GOLDMAN, R.C. & LEIVE, L. (1980) Heterogeneity of antigenic-side-chain length in lipopolysaccharide from *Escherichia coli* 0111 and *Salmonella typhimurium* LT2. Eur. J. Biochem. 107, 145-153.

GOLDSTEIN, I.M. (1985) Host factors in pathogenesis: the complement system-potential pathogenetic role in sepsis. In Septic Shock, pp 41-60. Edited by R.K. Root & M.A. Sande. Churchill Livingstone, New York.

GOLENBOCK, D.T., WILL, J.A., RAETZ, C.R.H. & PROCTOR, R.A. (1987) Lipid X ameliorates pulmonary hypertension and protects sheep from death due to endotoxin. Infect. Immun. 55, 2471-2476.

GOTSCHLICH, E.C., FRASER, B.A., NISHIMURA, O., ROBBINS, J.B. & LIU, T.-Y. (1981) Lipid on capsular polysaccharides of Gram-negative bacteria. J. Biol. Chem. 256, 8915-8921.

GOTTESMAN, S. (1984) Bacterial regulation: global regulatory networks. Annu. Rev. Genetics. 18, 415-441.

GOVAN, J.R.W. (1975) Mucoid strains of *Pseudomonas aeruginosa*: the influence of culture medium on the stability of mucus production. J. Med. Microbiol. 8, 513-522.

GOVAN, J.R.W. (1990) Characteristics of mucoid *Pseudomonas aeruginosa in vitro* and *in vivo*. In *Pseudomonas* Infection and Alginates. Biochemistry, genetics and pathology, pp 50-75. Edited by Gacesa & N.J. Russell. Chapman & Hall, London.

GOVAN, J.R.W. & FYFE, J.A.M. (1978) Mucoid *Pseudomonas aeruginosa* and cystic fibrosis: resistance of the mucoid form to carbenicillin, flucloxacillin and tobramycin and the isolation of mucoid variants *in vitro*. J. Antimicrob. Chemother. 4, 233-240.

GRANSDEN, W.R., EYKYN, S.J., PHILLIPS, I. & ROWE, B. (1990) Bacteremia due to *Escherichia coli*: a study of 861 episodes. Rev. Infect. Dis. 12, 1008-1018.

GRANSTRÖM, M., WRETLIND, B., MARKHAM, B. & CRYZ, S.J., Jr. (1988) Enzyme-linked immunosorbent assay to evaluate the immunogenicity of a polyvalent *Klebsiella* capsular polysaccharide vaccine in humans. J. Clin. Microbiol. 26, 2257-2261.

GREISMAN, E.S., DUBUY, B.J. & WOODWARD, C.L. (1978) Experimental Gram-negative bacterial sepsis: re-evaluation of the ability of rough mutant antisera to protect mice. Proc. Soc. Exp. Biol. Med. 158, 482-490.

GREISMAN, S.E., DUBUY, B.J. & WOODWARD, C.L. (1979) Experimental Gram-negative bacterial sepsis: prevention of mortality not preventable by antibiotics alone. Infect. Immun. 25, 538-557.

GREISMAN, S.E. & HORNICK, R.B. (1973) Mechanisms of endotoxin tolerance with special reference to man. J. Infect. Dis. 128, (Suppl.), 265-276.

GRIFFITHS, E. (1983) Availability of iron and survival of bacteria in infection. In Medical Microbiology, Vol.3, pp 153-177. Edited by C.S.F. Easmon, J. Jeljasewicz, M.R.W. Brown & P.A. Lambert, Academic Press, London & New York.

GRIFFITHS, E. (1987) The iron uptake systems of pathogenic bacteria. In Iron and infection-Molecular, Physiological and Clinical Aspects, pp 69-138. Edited by J. Bullen & E. Griffiths. John Wiley, Chichester.

GUESDON, J.-L., TERNYNCK, T. & AVRAMEAS, S. (1979) The use of avidin-biotin interaction in immunoenzymatic techniques. J. Histochem. Cytochem. 27, 1131-1139.

GUPTA, D.S., JANN, B. & JANN, K. (1984) *Escherichia coli* 018ac antigen: structure of the O-specific polysaccharide moiety. Infect. Immum. 45, 203-209.

GUTTENBERG, T.J., RØKKE, O., ANDERSEN, O. & JØRGENSEN, T. (1989) Early fall of circulating iron and rapid rise of lactoferrin in septicemia and endotoxemia: an early defence mechanism. Scand. J. Infect. Dis. 21, 709-715.

HABEEB, A.F.S.A. (1966) Determination of free amino groups in proteins by trinitrobenzenesulfonic acid. Anal. Biochem. 14, 328-336.

HACK, C.E., DE GROOT, E.R. & FELT-BERSMA R.J.F. (1989) Increased plasma levels of interleukin 6 in sepsis. Blood 74, 1704-1710.

HAMMERLING, G., LÜDERITZ, O. & WESTPHAL, O. (1971) Investigation on the core polysaccharide of *E. coli* 0100. Eur. J. Biochem. 22, 331-344.

HAMMOND, S.M., LAMBERT, P.A. & RYCROFT, A.N. (1984) The bacterial cell surface. Croom Helm, London.

HANCOCK, I.C. & POXTON, I.R. (1988) Bacterial Cell Surface Techniques. John Wiley & Sons, Chichester.

HANCOCK R.E.W. (1984) Alterations in outer membrane permeability. Ann. Rev. Microbiol. 38, 237-264.

HANCOCK, R.E.W., MUTHARIA, L.M., CHAN, L., DARVEAU, R.P., SPEERT, D.P. & PIER, G.B. (1983) *Pseudomonas aeruginosa* isolates from patients with cystic fibrosis: a class of serum-sensitive, nontypable strains deficient in lipopolysaccharide O side chains. Infect. Immun. 42, 170-177.

HARDER, W. & DIJKHUIZEN, L. (1983) Physiological responses to nutrient limitation. Ann. Rev. Microbiol. 37, 1-23.

HARDY, R.R. (1986) Purification and characterization of monoclonal antibodies. In Handbook of Experimental Immunology: 1. Immunochemistry, pp 13.1-13.13. Edited by D.M. Weir, L.A. Herzenberg, C. Blackwell & L.A. Herzenberg. Blackwell Scientific Publications, Oxford.

HARKONEN, S., SCANNON, P., MISCHAK, R.P., SPITLER, L.E., FOXALL, C., KENNEDY, D. & GREENBERG, R. (1987) Phase I study of a murine monoclonal anti-lipid A antibody in bacteremic and nonbacteremic patients. Antimicrob. Agents Chemother. 32, 710-716.

HARLOW, E. & LANE, D. (1988) Antibodies: A laboratory mannual. Cold Spring Harbor Laboratory, New York.

HEUMANN, D., BAUMGARTNER, J.D., JACOT-GUILLARMOD, H. & GLAUSER, M.P. (1991) Antibodies to core lipopolysaccharide determinants: absence of cross-reactivity with heterologous lipopolysaccharides. J. Infect. Dis. 163, 762-768.

HINCKLEY, A., MÜLLER, E. & ROTHFIELD, L. (1972) Reassembly of a membrane-bound multienzyme system. J. Biol. Chem. 247, 2623-2628.

HITCHCOCK, P.J. & BROWN, T.M. (1983) Morphological heterogeneity among *Salmonella* lipopolysaccharide chemotypes in silver stained polyacrylamide gels. J. Bacteriol. 154, 269-277.

HITCHCOCK, P.J. & MORRISON, D.C. (1984) The protein component of bacterial endotoxins. In Handbook of Endotoxin, Vol. 1: Chemistry of Endotoxin, pp 339-375. Edited by E.T. Rietschel. Elsevier, Amsterdam.

HOEKSTRA, D., VAN DER LAAN, J.W., DE LEIJ, L. & WIT, B. (1976) Release of outer membrane fragments from normally growing *Escherichia coli*. Biochem. Biophys. Acta 455, 889-899.

HOMMA, J.Y., MATSUURA, M., KANEGASAKI, S., KAWAKUBO, Y., KOJIMA, Y., SHIBUKAWA, N., KUMAZAWA, Y., YAMAMOTO, A., TANAMOTO, K., YASUDA, T., IMOTO, M., YOSHIHURA, H., KUSUMOTO, S. & SHIBA, T. (1985) Structural requirements of lipid A responsible for the functions: a study with chemically synthesized lipid A and its analogues. J. Biochem. 98, 395-406.

HORWITZ, M.A. & SILVERSTEIN S.C. (1980) Influence of the *Escherichia coli* capsule on complement fixation and on phagocytosis and complement killing by human phagocytes. J. Clin. Invest. 65, 82-94.

ISHIGURO, E.E., VANDERWEL, D. & KUSSER, W. (1986) Control of lipopolysaccharide biosynthesis and release by *Escherichia coli* and *Salmonella typhimurium*. J. Bacteriol. 168, 328-333.

ISPAHANI, P., PEARSON, N.J. & GREENWOOD, D. (1987) An analysis of community and hospital-acquired bacteraemia in a large teaching hospital in the United Kingdom. Q.T. Med., New Series. 63, 427-440.

JACOB, A.I., GOLDBERG, P.K., BLOOM, N., DEGENSTEIN, A. & KOZINN, P.J. (1977) Endotoxin and bacteria in portal blood. Gastroenterology 72, 1268-1270.

JANN, B. & JANN, K. (1990) Structure and biosynthesis of the capsular antigens of *Escherichia coli*. Curr. Top. Microbiol. Immunol. 150, 20-42.

JANN, K. & JANN, B. (1983) The K antigens of *Escherichia coli*. Prog. Allergy. 33, 53-79.

JANN, K. & JANN, B. (1984) Structure and biosynthesis of O-antigens. In Handbook of Endotoxin. Vol. 1. Chemistry of endotoxin, pp 138-186. Edited by E.Th. Rietschel. Elsevier, Amsterdam.

JANN, K. & JANN, B. (1985) Cell surface components and virulence: *Escherichia coli* O and K antigens in relation to virulence and pathogenicity. In The Virulence of *Escherichia coli*, pp 157-176. Edited by M. Sussman. Academic Press, London.

JANN, K. & JANN, B. (1987) Polysaccharide Antigens of *Escherichia coli*. Rev. Infect. Dis. 9, Suppl. 5, S517-S526.

JANSSON, P.E., LINDBERG, A.A., LINDBERG, B. & WOLLIN, R. (1981) Structural studies on the hexose region of the core in lipopolysaccharides from Enterobacteriaceae. Eur. J. Biochem. 115, 571-577.

JASPERS, L., MARGET, W., MAR, P.J., HOFFMAN, K., LANGECKER, P., RUCKDESCHEL, G., OBERMEIER, A. & KASTENBAUER, E. (1987) Antikorper gegen Lipoid A in der Bedandlung des septischen Schocks. Infection, 15, 146-152.

JENNINGS, H.J. (1983) Capsular polysaccharides as human vaccines. Adv. Carbohydr. Chem. Biochem. 41, 155-208.

JIMINEZ-LUCHO, V., JOINER, K.A., FOULDS, J., FRANK, M.M. & LEIVE, L. (1987) C3b generation is affected by the structure of O-antigen polysaccharide in lipopolysaccharide from Salmonella. J. Immunol. 139, 1253-1259.

JOHNS, M.A., SKEHILL, A., McCABE, W.R. (1983) Immunization with rough mutants of *Salmonella minnesota* IV. Protection by antisera to O and rough antigens against endotoxin. J. Infect. Dis. 147, 57-67.

JOHNSTON, C.A. & GREISMAN, S.E. (1985) Mechanisms of endotoxin tolerance. In Handbook of Endotoxin. Vol. 2. Pathophysiology of Endotoxin, pp 359-401. Edited by L.B. Hinshaw. Elsevier, Amsterdam.

JORGENSON, J.H. (1986) Clinical applications of the *Limulus* amebocyte lysate test. In Clinical Aspects of Endotoxin Shock, pp 127-160. Edited by R.A. Proctor. Elsevier, Amsterdam.

JUPIN, C., ANDERSON, S., DAMAIS, C., ALOUF, J.E. & PARANT, M. (1988) Toxic shock syndrome toxin 1 as an inducer of human tumor necrosis factors and gamma interferon. J. Exp. Med. 167, 752-761.

JUST, H.-M., METZGER, M., VOGEL, W. & PELKA, R.B. (1986) Treatment of intensive care unit patients with severe nosocomial infections. In Clinical use of intravenous immunoglobulins, pp 345-352. Edited by A. Morell, U.E. Nydegger. Academic Press, London.

KADURUGAMUWA, J.L., ANWAR, H., BROWN, M.R.W. & ZAK, O. (1985a) Protein antigens of encapsulated *Klebsiella pneumoniae* surface exposed after growth in the presence of subinhibitory concentrations of cephalosporins. Antimicrobial. Agents Chemother. 28, 195-199.

KADURUGAMUWA, J.L., ANWAR, H., BROWN, M.R.W. & ZAK, O. (1985b) Effect of subinhibitory concentrations of cephalosporins on surface properties and siderophore production in iron depleted *Klebsiella pneumoniae*. Antimicrob. Agents Chemother. 27, 220-223.

KAIJSER, B. & AHLSTEDT, S. (1977) Protective capacity of antibodies against *Escherichia coli* O and K antigens. Infect. Immun. 17, 286-289.

KAIJSER, B., HANSON, L.A., JODAL, V., LINDIN-JANSON, G. & ROBBINS, J.B. (1977) Frequency of *E. coli* K antigens in urinary tract infections in children. Lancet 1, 663-664.

KANEGASAKI, S., TANAMOTO, K., YASUDA, T., HOMMA, J.Y., MATSUURA, M., NAKATSUKA, M., KUMAZAWA, Y., YAMAMOTO, A., SHIBA, T., KUSUMOTO, S., IMOTO, M., YOSHIMURA, H. & SHIMAMOTO, T. (1986) Structure-activity relationship of lipid A: comparison of biological activities of natural and synthetic lipid A's with different fatty acid composition. J. Biochem. 99, 1203-1210.

KAPLAN, R., SAHN, S. & PETTY, T. (1979) Incidence and outcome of the respiratory distress syndrome in Gram-negative sepsis. Arch. Intern. Med. 139, 867-869.

KASPER, D.L., WINKELHAKE, J.L., ZOLLINGER, W.D., BRANDT, B.L. & ARTENSTEIN, M.S. (1973). Immunochemical similarity between polysaccharide antigens of *Escherichia coli* O7:K1L:NM and group B *Neisseria meningitidis*. J. Immunol. 110, 262-268.

KATZ, S., GROSFELD, J.L., GROSS, K., PLAGER, D.A., ROSS, D., ROSENTHAL, R.S., HULL, M. & WEBER, T.R. (1984) Impaired bacterial clearance and trapping in obstructive jaundice. Ann. Surg. 199, 14-20.

KAUFFMANN, B.M., CROSS, A.S., FUTROVSKY, S.L., SIDBERRY, H.F. & SADOFF, J.C. (1986) Monoclonal antibodies reactive with K1-encapsulated *Escherichia coli* lipopolysaccharide are opsonic and protective against lethal challenge. Infect. Immun. 52, 617-619.

KAUFFMANN, F. & VAHLNE, G. (1945) Über die Bedeutung des serologischen formenwechsels für die Bakteriophagenwirkung in der Coli-Gruppe. Acta Pathol. Microbiol. Scand. 22, 119-137.

KELLY, N.M., BELL, A. & HANCOCK, R.E.W. (1989) Surface characteristics of *Pseudomonas aeruginosa* grown in a chamber implant model in mice and rats. Infect. Immun. 57, 344-350.

KENDALL, C., IONESCU-MATIU, I. & DREASMAN, G.R. (1983) Utilization of the biotin/avidin system to amplify the sensitivity of the enzyme-linked immunosorbent assay (ELISA). J. Immunol. Methods 56, 329-339.

KENWARD, M.A. & BROWN, M.R.W. (1978) Relation between growth rate and sensitivity to cold shock of *Pseudomonas aeruginosa*. FEMS Microbiol. Lett. 3, 17-19.

KENWARD, M.A., BROWN, M.R.W. & FRYER, J.J. (1979) The influence of calcium or manganese on the resistance to EDTA, polymyxin B or cold shock, and the composition of *Pseudomonas aeruginosa* grown in glucose or magnesium depleted batch culture. J. Appl. Bacteriol. 47, 489-503.

KIENER, P.A., MAREK, F., RODGERS, G., LIN, P.F., WARR, G. & DESIDERIO, J. (1988) Induction of tumor necrosis factor, IFN-gamma and acute lethality in mice by toxic and non-toxic forms of lipid A. J. Immunol. 141, 870-874.

KILLION, J.W. & MORRISON, D.C. (1986) Protection of C3H/HeJ mice from lethal *Salmonella typhimurium* LT2 infection by immunization with lipopolysaccharide-lipid A-associated protein complexes. Infect. Immun. 54, 1-8.

KIM, K.S., KANG, J.H. & CROSS, A.S. (1986) The role of capsular antigens in serum resistance and *in vivo* virulence of *Escherichia coli*. FEMS Microbiol. Lett. 35, 275-278.

KIMURA, A. & HANSEN, E.J. (1986) Antigenic and phenotypic variations of *Haemophilus influenzae* type b lipopolysaccharide and their relationship to virulence. Infect. Immun. 51, 69-79.

KIPPS, T.J. & HERTZENBERG, L.A. (1986) Schemata for production of monoclonal antibody-producing hybridomas. In Handbook of Experimental Immunology: 4. Applications of Immunological Methods in Biomedical Sciences pp 108.1-108.9. Edited by D.M. Weir, L.A. Herzenberg, C. Blackwell & L.A. Herzenberg. Blackwell Scientific Publications, Oxford.

KIRKLAND, T.N., COLWELL, D.E., MICHALEK, S.M., McGHEE, J.R. & ZIEGLER, E.J. (1986) Analysis of the fine specificity and cross-reactivity of monoclonal anti-lipid A antibodies. J. Immunol. 137, 3614-3619.

KLUGER, M.J. & BULLEN, J.J. (1987) Clinical and physiological aspects. Idem. 243-282.

KOCHAN, I. (1977) Role of siderophores in nutritional immunity and bacterial parasitism. In Microorganisms and Minerals, pp 251-288. Edited by E.D. Weinberg, Dekker, New York.

KÖHLER, G. & MILSTEIN, C. (1975) Continuous cultures of fused cells secreting antibody of predefined specificity. Nature 256, 495-497.

KOIVURANTA-VARA, P., BANDA, D. & GOLDSTEIN, I.M. (1987) Bacterial lipopolysaccharide-induced release of lactoferrin from human polymorphonuclear leukocytes: role of monocyte-derived tumor necrosis factor. Infect. Immun. 55, 2956-2961.

KORHONEN, T.K., VALTONEN, M.V., PARKKINEN, J., VAISANEN-RHEN, V., FINNE, J., ØRSKOV, F., ØRSKOV, I., SVENSON, S.B. & MÄKELÄ, P.H. (1985) Serotypes, hemolysin production, and receptor recognition of *Escherichia coli* strains associated with neonatal sepsis and meningitis. Infect. Immun. 48, 486-491.

KREGER, B.E., CRAVEN, D.E., CARLING, P.C. & McCABE, W.R. (1980a) Gram-negative bacteremia III. Reassessment of etiology, epidemiology and ecology in 612 patients. Am. J. Med. 68, 332-343.

KREGER, B.E., CRAVEN, D.E. & McCABE, W.R. (1980b) Gram-negative bacteraemia. The evaluation of clinical features and treatment in 612 patients. Am. J. Med. 68, 344-355.

KRÖNCKE, K.-D., GOLECKI, J.R. & JANN, K. (1990b) Further electron microscopic studies on the expression of *Escherichia coli* Group 11 capsules. J. Bacteriol. 172, 3469-3472.

KRÖNCKE, K.-D., ØRSKOV, I., ØRSKOV, F., JANN, B. & JANN, K. (1990a) Electron microscopic study of coexpression of adhesive protein capsules and polysaccharide capsules in *Escherichia coli*. Infect. Immun. 58, 2710-2714.

KROPINSKI, A.M., CHAN, L.C. & MILAZZO, F.H. (1979) The extraction and analysis of lipopolysacchrides from *Pseudomonas aeruginosa* strain PAO, and three rough mutants. Can. J. Microbiol. 25, 390-398.

KROPINSKI, A.M., JEWELL, B., KUZIO, J., MILAZZO, F. & BERRY, D. (1985) Structure and functions of *Pseudomonas aeruginosa* lipopolysaccharide. Antibiot. Chemother. 36, 58-73.

KUSAMA, H. (1983) Enzyme-linked immunosorbent assay for detection of *Pseudomonas aeruginosa* lipopolysaccharides. J. Clin. Microbiol. 17, 317-322.

LACHMAN, E., PITSOE, S.B. & GAFFIN, S.L. (1984) Anti-lipopolysaccharide immunotherapy in management of septic shock of obstetric and gynaecological origin. Lancet I, 981-983.

LAEMMLI, U.K. (1970) Cleavage of structural proteins during the assembly of the head protein of bacteriophage T4. Nature 227, 680-685.

LEFFELL, M.S. & SPITZNAGEL, J.K. (1975) Fate of human lactoferrin and myeloperoxidase in phagocytizing human neutrophils: effects of immunoglobulin G subclasses and immune complexes coated on latex beads. Infect. Immun. 12, 813-820.

LEGAKIS, N.J., TZOOVELEKIS, L.S., MAKRIS, A. & KOTSIFAKI, H. (1989) Outer membrane alterations in multiresistant mutants of *Pseudomonas aeruginosa* selected by ciprofloxacin. Antimicrob. Agents Chemother. 33, 124-127.

LEGRAND, E.K. (1990) An evolutionary perspective of endotoxin: a signal for a well-adapted defense system. Med. Hypotheses 33, 49-56.

LEVIN, J., POORE, T.E., YOUNG, N.S., MARGOLIS, S., ZAUBER, N.P., TOWNES, A.S. & BELL, W.R. (1972) Gram-negative sepsis: detection of endotoxemia with the Limulus test. Ann. Intern. Med. 76, 1-7.

LEYING, H., SUERBAUM, S., KROLL, H.-P., STAHL, D. & OPFERKUCH, W. (1990) The capsular polysaccharide is a major determinant of serum resistance in K-1 positive blood culture isolates of *Escherichia coli*. Infect. Immun. 58, 222-227.

LIM, P.-K. (1990) A one-step two-particle latex immunoassay for the detection of *Salmonella typhi* endotoxin. J. Immunol. Methods 135, 257-261.

LIMET, J.N., BERBINSCHI, A., CLOECKAERT, A., CAMBIASO, C.L. & MASSON, P.L. (1988) Longitudinal study of brucellosis in mice by immunoassay of lipopolysaccharide antigens in blood and urine. J. Med. Microbiol. 26, 37-45.

LIU, P.V., MATSUMOTO, H., KUSAMA, H. & BERGAN, T. (1983) Survey of heat stable, major somatic antigens of *Pseudomonas aeruginosa*. Int. J. Syst. Bacteriol. 33, 256-264.

LIU, P.V. & WANG, S. (1990) Three new major somatic antigens of *Pseudomonas aeruginosa*. J. Clin. Microbiol. 28, 922-925.

LODGE, J.M.T., WILLIAMS, P. & BROWN, M.R.W. (1986) Influence of growth rate and iron limitation on the expression of outer membrane proteins and enterobactin by *Klebsiella pneumoniae* grown in continuous culture. J. Bacteriol. 165, 353-356.

LOPPNOW, H., BRADE, H., DÜRRBAUM, I., DINARELLO, S., KUSUMOTO, S., RIETSCHEL, E.TH. & FLAD, H.-D. (1989) IL1 induction capacity of defined lipopolysaccharide partial structures. J. Immunol. 142, 3229-3238.

LOPPNOW, H., LIBBY, P., FREUDENBERG, M., KRAUSS, J.H., WECKESSER, J. & MAYER, H. (1990) Cytokine induction by lipopolysaccharide (LPS) corresponds to lethal toxicity and is inhibited by nontoxic *Rhodobacter capsulatus* LPS. Infect. Immun. 58, 3743-3750.

LOW, D., ROBINSON, E.N.J., McGEE, Z.A. & FALKOW, S. (1987) The frequency of expression of pyelonephritis-associated pili is under regulatory control. Mol. Microbiol. 1, 335-346.

LOWRY, O.M., ROSEBROUGH, N.J., FARR, A.L. & RANDALL, R.J. (1951) Protein measurement with the folin-phenol reagent. J. Biol. Chem. 193, 265-275.

LUCE, J.M. (1987) Pathogenesis and management of septic shock. Chest 91, 883-888.

LÜDERITZ, O., FREUDENBERG, M.A., GALANOS, C., LEHMANN, V., RIETSCHEL, E.T. & SHAW, D.H. (1982) Lipopolysaccharides of Gram-negative bacteria. Curr. Top. Mem. Tran. 17, 79-151.

LÜDERITZ, O., STAUB, A.M. & WESTPHAL, O. (1966) Immunochemistry of O and R antigens of *Salmonella* and related *Enterobacteriaceae*. Bact. Rev. 30, 193-255.

LUGTENBERG, B. & VAN ALPHEN, L. (1983) Molecular architecture and functioning of the outer membrane of *Escherichia coli* and other Gram-negative bacteria. Biochim. Biophys. Acta 737, 51-115.

LUK, J.M.C. & LINDBERG, A.A. (1991) Rapid and sensitive detection of *Salmonella* (O:6,7) by immunomagnetic monoclonal antibody-based assays. J. Immunol. Methods 137, 1-8.

LUMSDEN, A.B., HENDERSON, J.M. & KUTNER, M.H. (1988) Endotoxin levels measured by a chromogenic assay in portal, hepatic, and peripheral venous blood in patients with cirrhosis. Hepatology 8, 232-236.

LUTTON, D.A., PATRICK, S., CROCKARD, A.D., STEWART, L.D., LARKIN, M.J., DERMOTT, E. & McNEILL, T.A. (1991) Flow cytometric analysis of within-strain variation in polysaccharide expression by *Bacteroides fragilis* by use of murine monoclonal antibodies. J. Med. Micro. 35, 229-237.

McCABE, W.R., DeMARIA, A., BERBERICH, H. & JOHNS, M.A. (1988) Immunization with rough mutants of *Salmonella minnesota*: Protective activity of IgM and IgG antibody to the R595 (Re chemotype) mutant. J. Infect. Dis. 158, 291-300.

McCABE, W.R., & JACKSON, G.G. (1962) Gram-negative bacteremia I. Etiology and ecology. Arch. Intern. Med. 110, 847-855.

McCABE, W.R., KAIJSER, B., OLLING, S., UWAYDAH, M. & HANSON, L.A. (1978) *Escherichia coli* in bacteremia: K and O antigens and serum sensitivity of strains from adults and neonates. J. Infect. Dis. 138, 33-41.

McCABE, W.R., KREGER, B.E. & JOHNS, M. (1972) Type-specific and cross-reactive antibodies in Gram-negative bacteremia. N. Eng. J. Med. 287, 261-267.

McCALLUS, D.E. & NORCROSS, N.L. (1987) Antibody specific for *Escherichia coli* J5 cross-reacts to various degrees with an *Escherichia coli* clinical isolate grown for different lengths of time. Infect. Immun. 55, 1042-1046.

McCONNELL, J.S. & COHEN, J. (1986) Release of endotoxin from *Escherichia coli* by quinolones. J. Antimicrob. Chemother. 18, 765-766.

McCONNELL, M. & WRIGHT, A. (1979) Variation in the structure and bacteriophage-inactivating capacity of *Salmonella anatum* lipopolysaccharide as a function of growth temperature. J. Bacteriol. 137, 746-751.

McCRACKEN, G.H. & MIZE, S.G. (1976) A controlled study of intrathecal antibiotic therapy in Gram-negative enteric meningitis in infancy. J. Pediatr. 89, 66-72.

McCUSKEY, R.S., McCUSKEY, P.A., URBASCHEK, R. & URBASCHEK, B. (1987) Küpffer cell function in host defence. Rev. Infect. Dis. 9, (Suppl. 5), S616-S619.

McCUTCHAN, J.A. & ZIEGLER, E.J. (1983) Treatment with anti-Gram-negative antibodies. Lancet II, 802-803.

McGOWAN, J.E., BARNES, M.W. & FINLAND, M. (1975) Bacteremia at Boston City Hospital: occurrence and mortality during 12 selected years (1935-1972), with special reference to hospital-acquired cases. J. Infect. Dis. 132, 316-335.

McGROARTY, E.J. & RIVERA, M. (1990) Growth-dependent alterations in production of serotype-specific and common antigen lipopolysaccharides in *Pseudomonas aeruginosa* PAO1. Infect. Immun. 58, 1030-1037.

McPHADEN, A.R. & WHALEY, K. (1985) The complement system in sepsis and trauma . Br. Med. Bull. 41, 281-286.

MANDINE, E., SALLES, M.-F., ZALISZ, R., GUENOUNOU, M. & SMETS, P. (1990) Murine monoclonal antibodies to *Klebsiella pneumoniae* protect against lethal endotoxaemia and experimental infection with capsulated *K. pneumoniae*. Infect. Immun. 58, 2828-2833.

MANNEL, D.N., MOORE, R.N. & MERGENHAGEN, S.E. (1980) Macrophages as a source of tumoricidal activity (tumor-necrotizing factor) Infect. Immun. 30, 523-530.

MANSOUR, J.D., ROBSON, J.A., ARNDT, C.W. & SCHULTE, T.H. (1985) Detection of *Escherichia coli* in blood using flow cytometry. Cytometry 6, 186-190.

MARCUS, R.L., SHIN, H.S., MAYER, M.M. (1971) An alternative pathay: C3 clearing activity not due to C42a on endotoxic lipopolysaccharide after treatment with guinea pig serum: relation to properdin (complement components). Proc. Natl. Acad. Sci. USA 68, 1351.

MARGET, W., MAR, P.J., JASPERS, L., POSSINGER, K. & HASLBERGER, H. (1985) Preliminary study on administration of high-titer Lipid A antibody serum in sepsis and septic shock patients. Infection 13, 120-124.

MARKS, M.I., ZIEGLER, E.J., DOUGLAS, H., CORBEIL, L.B., BRAUDE, A.I. (1982) Induction of immunity against lethal *Haemophilus influenzae* type b infection by *Escherichia coli* core lipopolysaccharide. J. Clin. Invest. 69, 742-749.

MARTIN, N.L. & BEVERIDGE T.J., (1986) Gentamicin interaction with *Pseudomonas aeruginosa* cell envelope. Antimicrob. Agents Chemother. 29, 1079-1087.

MASON, A.D., Jr., McMANUS, A.T. & PRUITT, B.A. (1986) Association of burn mortality and bacteraemia: a 25-year review. Arch. Surg. 121, 1027-1031.

MATHISON, J.C. & ULEVICH, R.J. (1981) *In vivo* interaction of bacterial lipopolysaccharide (LPS) with rabbit platelets: modulation by C3 and high density lipoproteins. J. Immunol. 126, 1575-1580.

MATHISON, J.C., WOLFSON, E. & ULEVITCH, R.J. (1988) Participation of tumor necrosis factor in the mediation of Gram-negative bacterial lipopolysaccharide-induced injury in rabbits. J. Clin. Invest. 81, 1925-1937.

MATSUURA, M. & GALANOS, C. (1990) Induction of hypersensitivity to endotoxin and tumor necrosis factor by sublethal infection with *Salmonella typhimurium*. Infect. & Immun. 58, 935-937.

MATSUURA, M., YAMAMOTO, A., KOJIMA, Y., HOMMA, J.Y., KISO, M. & HASEGAWA, A. (1985) Biological activities of chemically synthesized partial structural analogues of lipid A. J. Biochem. 98, 1229-1237.

MATTSBY-BALTZER, I., GEMSKI, P. & ALVING, C.R. (1984) Heterogeneity of lipid A: comparison of lipid A types from different Gram-negative bacteria. J. Bacteriol. 159, 900-904.

MAYORAL, J.L. & DUNN, D.L. (1990) Cross-reactive murine monoclonal antibodies directed against the core/lipid A region of endotoxin inhibit production of tumor necrosis factor. J. Sur. Res. 49, 287-292.

MEERS, J.L. & TEMPEST, D.W. (1968) The influence of extracellular products on the behaviour of mixed microbial populations in magnesium-limited chemostat cultures. J. Gen. Micro. 52, 309-317.

MEHTA, N.D., WILSON, B.M., RAPSON, N.T. & EASMON, C.S.F. (1988) Comparison of the opsonic activity of polyclonal and monoclonal antibodies raised against *Salmonella minnesota* R595. J. Med. Micro. 25, 85-93.

MEHTA, N.D., WILSON, B.M., RAPSON, N.T. & EASMON, C.S.F. (1990) A comparison of specificity and biological activity of polyclonal and monoclonal antibodies raised against *Salmonella minnesota* R595 lipopolysaccharide. J. Med. Micro. 31, 85-93.

MERTSOLA, J., MUNFORD, R.S., RAMILO, O., SÁEZ-LLORENS, X., MUSTAFA, M.M., McCRACKEN, G.H., Jr. & HANSEN, E.J. (1990) Specific detection of *Haemophilus influenzae* type b lipooligosaccharide by immunoassay. J. Clin. Microbiol. 28, 2700-2706.

MERTSOLA, J., RAMILO, O., SÁEZ-LLORENS, X., HANSON, M.S., McCRACKEN, G.H. & HANSEN, E.J. (1989) Specific detection of *Haemophilus influenzae* type b lipooligosaccharide by a polymyxin B monoclonal antibody assay. J. Immunol. Methods 122, 219-226.

MILLER, J.F., MEBALANOS, J.J. & FALKOW, S. (1989) Co-ordinate regulation and sensory transduction in the control of bacterial virulence. Science 243, 916-922.

MINAH, G.E., REDNOR, J.L., PETERSON, D.E., OVERBOLSER, C.D., DE PAOLA, L.G. & SUZUKI, J.B. (1986) Oral succession of Gram-negative bacilli in myelosuppressed patients. J. Clin. Microbiol. 24, 210-213.

MINER, K.M., MANYAK, C.L., WILLIAMS, E., JACKSON, J., JEWELL, M., GAMMON, M.T., EHRENFREUND, C., HAYES, E., CALLAHEN, L.T., III., ZWEERINK, H. & SIGAL, N.H. (1986) Characterization of murine monoclonal antibodies to *Escherichia coli* J5. Infect. Immun. 52, 56-62.

MOODY, M.R., YOUNG, V.M., KEMTON, D.M. & VERMEULEN, G.D. (1972) *Pseudomonas aeruginosa* in a center for cancer research. I. Distribution of intraspecies types from human and environmental sources. J. Infect. Dis. 125, 95-101.

MORRISON, D.C. (1989) The case for specific lipopolysaccharide receptors expressed on mammalian cells. Microb. Pathogen. 7, 389-398.

MORRISON, D.C. & JACOBS, D.M. (1976) Binding of polymyxin B to the lipid A portion of bacterial lipopolysaccharides. Immunochem. 13, 813-818.

MORRISON, D.C. & KLINE, L.F. (1977) Activation of the classical and properdin pathways of complement by bacterial lipopolysaccharides (LPS). J. Immunol. 118, 362-370.

MORRISON, D.C. & RYAN, J.L. (1987) Endotoxins and disease mechanisms. Ann. Rev. Med. 38, 417-432.

MORRISON, D.C., SILVERSTEIN, R., BRIGHT, S.W., CHEN, T.-Y., FLEBBE, L.M. & LEI, M.-G. (1990) Monoclonal antibody to mouse lipopolysaccharide receptor protects mice against the lethal effects of endotoxin. J. Infect. Dis. 162, 1063-1068.

MORRISON, D.C. & ULEVITCH, R.J. (1978) The effects of bacterial endotoxins on host mediation systems. Am. J. Pathol. 93, 527-618.

MORRISON, D.C., VUKAJLOVICH, S.W., GOODMAN, S.A. & WOLLENWEBER, H.-W. (1985) Regulation of lipopolysaccharide biologic activity by polysaccharide: In Bayer-Symposium VIII: The Pathogenesis of Bacterial Infections, pp 68-84. Edited by G.G. Jackson & H. Thomas, Springer-Verlag, Berlin.

MOXON, E.R. & KROLL, J.S. (1990) The role of bacterial polysaccharide capsules as virulence factors. Curr. Top. Microbiol. Immunol. 150, 65-85.

MÓZES, T., BEN-EFRAIM, S., TAK, C.J.A.M., HEILIGERS, J.P.C., SAXENA, P.R. & BONTA, I.L. (1991) Serum levels of tumor necrosis factor determine the fatal or non-fatal course of endotoxic shock. Immunol. Letts. 27, 157-162.

MÜHLRADT, P.F. & GOLECKI, J.R. (1975) Asymmetrical distribution and artifactual reorientation of lipopolysaccharide in the outer membrane bilayer of *Salmonella typhimurium*. Eur. J. Biochem. 51, 343-352.

MUIRHEAD, K.A., HORAN, P.K. & POSTE, G. (1985) Flow cytometry: present and future. Biotechnology 3, 337-356.

MUNFORD, R.S. & DIETSCHY, J.M. (1985) Effects of specific antibodies, hormones, and lipoproteins on bacterial lipopolysaccharides injected into the rat. J. Infect. Dis. 152, 177-184.

MUNFORD, R.S., HALL, C.L. & GRIMM, L. (1984) Detection of free endotoxin in cerebrospinal fluid by the *Limulus* lysate test. Infect. Immun. 45, 531-533.

MUNFORD, R.S., HALL, C.L., LIPTON, J.M. & DIETSCHY, J.M. (1982) Biological activity, lipoprotein-binding behaviour, and *in vivo* disposition of extracted and native forms of *Salmonella typhimurium* lipopolysaccharides. J. Clin. Invest. 70, 877-888.

MUNFORD, R.S., HALL, C.L. & RICK, P.D. (1980) Size heterogeneity of *Salmonella typhimurium* lipopolysaccharides in outer membranes and culture supernatant membrane fragments. J. Bacteriol. 144, 630-640.

MUTHARIA, L.M., CROCKFORD, G., BOGARD, W.C., Jr. & HANCOCK, R.E.W. (1984) Monoclonal antibodies specific for *Escherichia coli* J5 lipopolysaccharide: cross-reaction with Gram-negative bacterial species. Infect. Immun. 45, 631-636.

NAGACHINTA, T., STEPHENS, M., REITZ, B. & POLK, B.F. (1987) Risk factors for surgical-wound infection following cardiac surgery. J. Infect. Dis. 156, 967-973.

NAKAE, T. (1986) Outer membrane permeability of bacteria. In CRC Critical Reviews in Microbiology. Vol. 13, pp 1-56. CRC Press Inc., New York.

NATANSON, C., DANNER, R.L., ELIN, R.J., HOSSEINI, J.M., PEART, K.W., BANKS, S.M., MacVITTIE, T.J., WALKER, R.I. & PARRILLO, J.E. (1989) Role of endotoxemia in cardiovascular dysfunction and mortality. *Escherichia coli* and *Staphylococcus aureus* challenges in a canine model of human septic shock. J. Clin. Invest. 83, 243-251.

NEILANDS, J.B. (1981) Microbial iron compounds. Ann. Rev. Biochem. 50, 715-731.

NEILANDS, J.B. (1982) Microbial envelope proteins related to iron. Ann. Rev. Microbiol. 36, 285-309.

NEILSON, I.R., NEILSON, A., YUNIS, E.J. & ROWE, M.I. (1989) Failure of tumor necrosis factor to produce hypotensive shock in the absence of endotoxin. Surgery 106, 439-443.

NELLES, M.J. & NISWANDER, C.A. (1984) Mouse monoclonal antibodies reactive with J5 lipopolysaccharide exhibit extensive serological cross-reactivity with a variety of Gram-negative bacteria. Infect. Immun. 46, 677-681.

NELSON, J.W. (1990) Pulmonary colonisation of patients with cystic fibrosis by *Pseudomonas aeruginosa*. PhD thesis, University of Edinburgh.

NEU, H.C., FRACARO, M., BOPP, H., O'KEEFE, M. & O'CONNOR, J. (1990) Bacteraemia - a New York perception. J. Antimicrob. Chemother. 25, Suppl. C. 107-113.

NG, A.K., CHEN, C.L.H., CHANG, C.M. & NOWOTNY, A. (1976) Relationship of structure to function in bacterial endotoxins: serologically cross-reactive components and their effect on protection of mice against some Gram-negative infections. J. Gen. Microbiol. 94, 107-116.

NICAS, T.L. & HANCOCK, R.E.W. (1980) Outer membrane protein H1 of *Pseudomonas aeruginosa*: involvement in adaptive and mutational resistance to ethylenediaminetetraacetate, polymyxin B and gentamicin. J. Bacteriol. 143, 872-878.

NIKAIDO, H. & NAKAE, T. (1979) The outer membrane of Gram-negative bacteria. Adv. Microbiol. Physiol. 20, 163-250.

NIKAIDO, H. & VAARA, M. (1985) Molecular basis of bacterial outer membrane permeability. Microbiol. Rev. 49, 1-32.

NISHIJIMA, M., BULAWA, C.E. & RAETZ, C.R.H. (1981) Two interacting mutations causing temperature-sensitive phosphatidylglycerol synthesis in *Escherichia coli* membranes. J. Bacteriol. 145, 113-121.

NISHIJIMA, M. & RAETZ, C.R.H. (1981) Characterization of two membrane-associated glycolipids from an *Escherichia coli* mutant deficient in phosphatidylglycerol. J. Biol. Chem. 256, 10690-10696.

NIXON, B.T., RONSON, C.W. & AUSUBEL, F.M. (1986) Two-component regulatory systems responsive to environmental stimuli share strongly conserved domains with the nitrogen assimilation regulatory genes ntrB and ntrC. Proc. Natl. Acad. Sci. USA 83, 7850-7854.

NOLAN, J.P. (1975) The role of intestinal endotoxins in liver injury. Gastroenterology 69, 1346-1356.

NOLAN, J.P. (1990) Detection of bacterial endotoxins: another step forward. J. Lab. Clin. Med. 116, 140-141.

NYS, M., JOASSIN, L., SOMZEE, A. & DEMONTY, J. (1988) Enzyme-linked immunosorbent assay for immunoglobulin G subclass antibodies specific for Enterbacterial Re core glycolipid in healthy individuals and in patients infected by Gram-negative bacteria. J. Clin. Microbiol. 26, 857-862.

OBAYASHI, T., TAMURA, H., TANAKA, S., OHKI, M., TAKAHASHI, S. & KAWAI, T. (1986) Endotoxin-inactivating activity in normal and pathological human blood samples. Infect. Immun. 53, 294-297.

OPAL, S.M., CROSS, A.S., KELLY, N.M., SADOFF, J.C., BODMER, M.W., PALARDY, J.E. & VICTOR, G.H. (1990) Efficacy of a monoclonal antibody directed against tumor necrosis factor in protecting neutropenic rats from lethal infection with *Pseudomonas aeruginosa*. J. Infect. Dis. 161, 1148-1152.

ØRSKOV, F., ØRSKOV, I., EVANS, D.J., Jr., SACK, R.B. & WADSTROM, T. (1976) Special *Escherichia coli* serotypes among enterotoxigenic strains from diarrhoea in adults and children. Med. Microbiol. Immunol. 162, 73-78.

ØRSKOV, F., ØRSKOV, I., SUTTON, A., SCHNEERSON, R., LIN, W., EGAN, W., HOFF, G.E. & ROBBINS, J.B. (1979) Form variation in *Escherichia coli* K1: determined by O-acetylation of the capsular polysaccharide. J. Exp. Med. 149, 669-685.

ØRSKOV, F., SHARMA, V. & ØRSKOV, I. (1984) Influence of growth temperature on the development of *Escherichia coli* polysaccharide K antigens. J. Gen. Microbiol. 130, 2681-2684.

ØRSKOV, F., WHITTAM, T.S., CRAVIOTO, A. & ØRSKOV, I. (1990) Clonal relationships among classic enteropathogenic *Escherichia coli* (EPEC) belonging to different O groups. J. Infect. Dis. 162, 76-81.

ØRSKOV, I., BIRCH-ANDERSON, A., DUGUID, J.P., STENDERUP, J. & ØRSKOV, F. (1985) An adhesive protein capsule of *Escherichia coli*. Infect. Immun. 47, 191-200.

ORSKOV, I. & ORSKOV, F. (1985) *Escherichia coli* extraintestinal infections. J. Hyg. 95, 551-575.

ØRSKOV, I., ØRSKOV, F., JANN, B. & JANN, K. (1977) Serology, chemistry and genetics of O and K antigens of *Escherichia coli*. Bacteriol. Rev. 41, 667-710.

OSBORN, M.J. (1969) Structure and biosynthesis of the bacterial cell wall. Ann. Rev. Biochem. 38, 501-538.

OSBORN, M.J., GANDER, J.E., PARISI, E. & CARSON, J. (1972) Mechanism of assembly of the outer membrane of *Salmonella typhimurium*: isolation and characterization of cytoplasmic and outer membrane. J. Biol. Chem. 247, 3962-3972.

OVERBEEK, B.P., DE VOS, N.M., KELLER, N., RAPONI, G., ROZENBERG-ARSKA, M. & VERHOEF, J. (1989). Enhanced binding of murine monoclonal antibodies to lipopolysaccharide structures of *Enterobacteriaceae* after treatment with antibiotics. Serodiag. Immunother. Infect. Dis. 4, 39-51.

OVERBEEK, B.P., SCHELLEKENS, J.F.P., LIPPE, W., DEKKER, B.A.T. & VERHOEF, J. (1987) Carumonam enhances reactivity of *Escherichia coli* with mono- and polyclonal antisera to rough mutant *Escherichia coli* J5. J. Clin. Micro. 25, 1009-1013.

OVERBEEK, B.P. & VERINGA, E.M. (1991) Role of antibodies and antibiotics in aerobic Gram-negative septicaemia: possible synergism between antimicrobial treatment and immunotherapy. Rev. Infect. Dis. 13, 751-760.

PABST, H.J. & JOHNSTON, R.B. (1980) Increased production of superoxide anion by macrophages exposed *in vitro* to muramyl dipeptide or lipopolysaccaride. J. Exp. Med. 151, 101-114.

PAGANI, L., LANDINI, L., LUZZARO, F., DEBIUGGI, M. & ROMERO, E. (1990) Emergence of cross-resistance to imipenem and other β-lactam antibiotics in *Pseudomonas aeruginosa* during therapy. Microbiologica 13, 43-55.

PALVA, L. & MÄKELÄ, P.H. (1980) Lipopolysaccharide heterogeneity in *Salmonella typhimurium* analyzed by sodium dodecyl sulfate/ polyacrylamide gel electrophoresis. Eur. J. Biochem. 107, 137-143.

PARANT, M., PARANT, F. & CHEDID, L. (1977) Inheritance of lipopolysaccharide-enhanced non-specific resistance to infection and of susceptibility to endotoxic shock in lipopolysaccharide low-responder mice. Infect. Immun. 16, 432-438.

PARHAM, P. (1986) Preparation and purification of active fragments from mouse monoclonal antibodies. In Handbook of Experimental Immunology: I. Immunochemistry pp 14.1-14.23. Edited by D.M. Weir, L.A. Herzenberg, C. Blackwell & L.A. Herzenberg. Blackwell Scientific Publications, Oxford.

PARRILLO, J.E., PARKER, M.M., NATANSON, C., SUFFREDINI, A.F., DANNER, R.L., CUNNION, R.E. & OGNIBENE, F.P. (1990). Septic shock in humans: advances in the understanding of pathogenesis, cardiovascular dysfunction, and therapy. Ann. Intern. Med. 113, 227-242.

PATRICK, S., REID, J.H. & LARKIN, M.J. (1984) The growth and survival of capsulate and non-capsulate *Bacteroides fragilis in vivo* and *in vitro*. J. Med. Micro. 17, 237-246.

PELTOLA, H., SALOMMA, T., SIVONEN, A. & RENKONEN, O.-V. (1987) Septicaemia in a University pediatric hospital: a five year analysis. Scand. J. Infect. Dis. 19, 277-282.

PENKETH, A., PITT, T., ROBERTS, D., HODSON, M.E. & BATTEN, J.C. (1983) The relationship of phenotype changes in *Pseudomonas aeruginosa* to the clinical condition of patients with *Pseudomonas aeruginosa*. Am. Rev. Respir. Dis. 127, 605-608.

PENNINGTON, J.E. & MENKES, E. (1981) Type-specific vs. cross-protective vaccination for Gram-negative bacterial pneumonia. J. Infect. Dis. 144, 599-603.

PERERA, V.Y., CREASY, M.T. & WINTER, A.J. (1983) Nylon bead enzyme-linked immunosorbent assay for detection of sub-picogram quantities of *Brucella* antigens. J. Clin. Microbiol. 18, 601-608.

PEREZ-PEREZ, G.I., HOPKINS, J.A. & BLASER, M.J. (1986) Lipopolysaccharide structures in Enterobacteriaceae, *Pseudomonas aeruginosa* and *Vibrio cholerae* are immunologically related to *Compylobacter* species. Infect. Immun. 51, 204-208.

PETER, G., PIZZO, P.A., ROBICHAUD, K., VISCONTI, E.B., FORMAN, E.N. & ZINNER, S.A. (1979) Possible protective effect of circulating antibodies to the shared core glycolipid (CGL) of Enterobacteriaeae in children with malignancy. Pediatr. Res. 13, 46.

PETERSON, A.A., HANCOCK, R.E.W. & McGROARTY, E.J. (1985) Binding of polycationic antibiotics and polyamines to lipopolysaccharides of *Pseudomonas aeruginosa*. J. Bacteriol. 164, 1256-1261.

PHILLIPS, A.P., MARTIN, K.L. & CAPEY, A.J. (1987) Direct and indirect immunofluorescence analysis of bacterial populations by flow cytometry. J. Immunol. Methods 101, 219-228.

PIKE, W.J., COCKAYNE, A., WEBSTER, C.A., SLACK, R.C.B. & ARBUTHNOTT, J.P. (1991) The development and design of a novel *in vivo* chamber implant for the analysis of microbial virulence and assessment of antimicrobial therapy. Society for general microbiology, 119th Meeting, Edinburgh. Abstract 1400.

PIROFSKY, B. (1984) Intravenous immune globulin therapy in hypogammaglobulinemia. A review. Am. J. Med. 76(3A), 53-60.

PLUSCHKE G. & ACHTMAN, M. (1985) Antibodies to O-antigen of lipopolysaccharide are protective against neonatal infection with *Escherichia coli* K1. Infect. Immun. 49, 365-370.

PLUSCHKE, G., MOLL, A., KUSACEK, B. & ACHTMAN, M. (1986) Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and monoclonal antibodies as tools for the subgrouping of *Escherichia coli* lipopolysaccharide O18 and O23 antigens. Infect. Immun. 51, 286-293.

POLLACK, M., CHIA, J.K.S., KOLES, N.L., MILLER, M. & GUELDE, G. (1989) Specificity and cross-reactivity of monoclonal antibodies reactive with the core lipid A region of bacterial lipopolysaccharide. J. Infect. Dis. 159, 168-184.

POLLACK, M., HUANG, A.I., PRESCOTT, R.K., YOUNG, L.S., HUNTER, K.W., CRUESS, D.F. & TSAI, C.-M. (1983) Enhanced survival in *Pseudomonas aeruginosa* septicemia associated with high levels of circulating antibody to *Escherichia coli* endotoxin core. J. Clin. Invest. 72, 1874-1881.

POLLACK, M., RAUBITSCHEK, A.A. & LARRICK, J.W. (1987) Human monoclonal antibodies that recognise conserved epitopes in the core-lipid A region of lipopolysaccharides. J. Clin. Invest. 79, 1421-1430.

PORAT, R., JOHNS, M.A. & McCABE, W.R. (1987) Selective pressures and lipopolysaccharide subunits as determinants of resistance of clinical isolates of Gram-negative bacilli to human serum. Infect. Immun. 55, 320-328.

POXTON, I.R. & ARBUTHNOTT, J.P. (1990) Determinants of bacterial virulence. In Topley & Wilsons Principles of Bacteriology, Virology and Immunity. Vol. 1, 8th Edition, pp 331-353. Edited by A.H. Linton & H.M. Dick. Edward Arnold, London.

POXTON, I.R., BELL, G.T. & BARCLAY, G.R. (1985) The association on SDS-polyacrylamide gels of lipopolysaccharide and outer membrane proteins of *Pseudomonas aeruginosa* as revealed by monoclonal antibodies and Western blotting. FEMS Microbiol. Lett. 27, 247-251.

PREHM, P., STIRM, S., JANN, B. & JANN, K. (1975) Cell-wall lipopolysaccharide from *Escherichia coli* B. Eur. J. Biochem. 56, 41-55.

PREHM, P., STIRM, S., JANN, B., JANN, K. & BONAN, H.G. (1976) Cell wall lipopolysaccharide of ampicillin-resistant mutants of *E. coli* K12. Eur. J. Biochem. 66, 369-377.

PRIEST, B.P., BRINSON, D.N., SCHROEDER, D.A. & DUNN, D.L. (1989) Treatment of experimental Gram-negative bacterial sepsis with murine monoclonal antibodies directed against lipopolysaccharide. Surgery, 106, 147-155.

PROCTOR, R.A., WILL, J.A., BURHOP, K.E. & RAETZ, C.R.H. (1986) Protection of mice against lethal endotoxemia by a lipid A precursor. Infect. Immun. 52, 905-907.

PYLE, S.W. & SCHILL, W.B. (1985) Rapid serological analysis of bacterial LPS by electrotransfer to nitrocellulose. J. Immunol. Methods 85, 371-382.

QURESHI, N., TAKAYAMA, K. & RIBI, E. (1982) Purification and structural determination of non-toxic lipid A obtained from the lipopolysaccharide of *Salmonella typhimurium*. J. Biol. Chem. 257, 11808-11815.

RAETZ, C.R.H. (1990) Biochemistry of endotoxins. Ann. Rev. Biochem. 59, 129-170.

RAPONI, G., KELLER, N., OVERBEEK, B.P., ROZENBERG-ARSKA, M., VAN KESSEL, K.P.M. & VERHOEFF, J. (1990) Enhanced phagocytosis of encapsulated *Escherichia coli* strains after exposure to sub-MICs of antibiotics is correlated to changes of the bacterial cell surface. Antimicrob. Agents Chemother. 34, 332-336.

RAPONI, G., KELLER, N., OVERBEEK, P., ROZENBERG-ARSKA, M., TORENSMA, R. & VERHOEF, J. (1991) Immunization of mice with antibiotic-treated *Escherichia coli* results in enhanced protection against challenge with homologous and heterologous bacteria. J. Infect. Dis. 163, 122-127.

RAVIN, H.A., ROWLEY, D., JENKINS, C. & FINE, J. (1960) On the absorption of bacterial endotoxin from the gastrointestinal tract of the normal and shocked animal. J. Exp. Med. 112, 783-792.

RAY, B.L., PAINTER, G. & RAETZ, C.R.H. (1984) The biosynthesis of Gram-negative endotoxin. J. Biol. Chem. 259, 4852-4859.

REMICK, D.G., KUNKEL, R.G., LARRICK, J.W. & KUNKEL, S.L. (1987) Acute *in vivo* effects of human recombinant tumor necrosis factor. Lab. Invest. 56, 583-590.

RICK, P.D., FUNG, L.W.-M., HO, C. & OSBORN, M.J. (1977) Lipid A mutants of *Salmonella typhimurium*. J. Biol. Chem. 252, 4904-4912.

RICK, P.D. & OSBORN, M.J. (1977) Lipid A mutants of *Salmonella typhimurium*. J. Biol. Chem. 252, 4895-4903.

RIETSCHEL, E.T., BRADE, L., BRANDENBURG, K., FLAD, H.-D., DE JONG-LEUVENINCK, J., KAWAHARA, K., LINDNER, B., LOPPNOW, H., LÜDERITZ, T., SCHADE, U., SEYDEL, U., SIDORCZYK, Z., TACKEN, A., ZÄHRINGER, U. & BRADE, H. (1987) Chemical structure and biologic activity of bacterial and synthetic lipid A. Rev. Infect. Dis. 9, (Suppl. 5), S527-S536.

RIETSCHEL, E.T., WOLLENWEBER, H.-W., BRADE, H., ZÄHRINGER, U., LINDNER, B., SEYDEL, U., BRADACZEK, H., BARNICKEL, G., LABISCHINSKI, H. & GIESBRECHT, P. (1984a) Structure and conformation of the lipid A component of lipopolysaccharides. In Handbook of Endotoxin. Vol. 1. Chemistry of Endotoxin, pp 187-220. Edited by E.T. Rietschel. Elsevier, Amsterdam.

RIETSCHEL, E.T., ZÄHRINGER, U., WOLLENWEBER, H.-W., MIRAGLIOTTA, G., MUSEHOLD, J., LÜDERITZ, T. & SCHADE, U. (1984b) Bacterial endotoxins: chemical structure and biological activity. Am. J. Emerg. Med. 2, 60-69.

RIFKIND, D. (1967) Prevention by polymyxin B of endotoxin lethality in mice. J. Bacteriol. 93, 1463-1464.

RIVERA, M., BRYAN, L.E., HANCOCK, R.E.W. & McGROARTY, E.J. (1988) Heterogeneity of lipopolysaccharides from *Pseudomonas aeruginosa*: analysis of lipopolysaccharide chain length. J. Bacteriol. 170, 512-521.

ROBBINS, J.B. (1978) Vaccines for the prevention of encapsulated bacterial diseases. Current status, problems and prospects for the future. Immunochem. 15, 839-854.

ROBERT-GERO, M., POIRET, M. & COHEN, G.N. (1970) The aspartate kinase of *Pseudomonas putida* regulation of synthesis and activity. Biochem. Biophys. Acta 206, 17-30.

ROBERTSON, B.R. & BUTTON, D.F. (1989) Characterizing aquatic bacteria according to population, cell size, and apparent DNA content by flow cytometry. Cytometry 10, 70-76.

ROBINSON, A. & TEMPEST, D.W. (1973) Phenotypic variability of the envelope proteins of *Klebsiella aerogenes*. J. Gen. Microbiol. 78, 361-370.

ROCKE, D.A., GAFFIN, S.L., WELLS, M.T., KOEN, Y. & BROCKE-UTNE, J.G. (1987) Endotoxaemia associated with cardiopulmonary bypass. J. Thorac. Cardiovasc. Surg. 93, 832-837.

RONSON, C.W., NIXON, B.T. & AUSUBEL, F.M. (1987) Conserved domains in bacterial regulatory proteins that respond to environmental stimuli. Cell 49, 579-581.

ROTH, R.I., LEVIN, F.C. & LEVIN, J. (1990) Optimization of detection of bacterial endotoxin in plasma with the Limulus test. J. Lab. Clin. Med. 116, 153-161.

ROTHFIELD, L. & ROMEO, D. (1971) Role of lipids in the biosynthesis of the bacterial cell envelope. Bacteriol. Rev. 35, 14-38.

ROTHSTEIN, J.L. & SCHREIBER, H. (1988) Synergy between tumor necrosis factor and bacterial products causes hemorrhagic necrosis and lethal shock in normal mice. Proc. Natl. Acad. Sci. 85, 607-611.

ROZENBERG-ARZKA, M., PORSUSI, J.C., JAARSMA, E.Y. & VERHOFF, J. (1986) Bactericidal, bacteriolytic and opsonic activity of human serum against *Escherichia coli*. J. Med. Micro. 22, 143-149.

RYAN, J.L. (1985) Microbial factors in pathogenesis: Lipopolysaccharides. In Septic Shock, pp 13-25. Edited by R.K. Root & M.A. Sande. Churchill Livingstone, New York.

SAKULRAMRUNG, R. & DOMINGUE, G.J. (1985) Cross-reactive immunoprotective antibodies to *Escherichia coli* O111 rough mutant J5. J. Infect. Dis. 151, 995-1004.

SALLES, M.-F., MANDINE, E., ZALISZ, R., GUENOUNOU, M. & SMETS, P. (1989) Protective effects of murine monoclonal antibodies in experimental septicaemia: *E. coli* antibodies protect against different serotypes of *E. coli*. J. Infect. Dis. 159, 641-647.

SAUKONEN, K.M.J., NOWICKI, B. & LEINONEN, M. (1988) Role of type 1 and S fimbriae in the pathogenesis of *Escherichia coli* O18:K1 bacteraemia and meningitis in the infant rat. Infect. Immun. 56, 892-897.

SCHADE, A.L. & CAROLINE, L. (1946) An iron-binding component in human blood plasma. Science 104, 340-341.

SCHEDEL, I. (1988) New aspects in the treatment of Gram-negative bacteraemia and septic shock. Infection 16, 4-7.

SCHELLEKENS, J., BROUWER, E., CALANDRA, Th., ROZENBERG-ARSKA, M. & VERHOEF, J. (1989) Humoral immunity in human Gram-negative septic shock. I. Potentially cross-reactive anti-lipopolysaccharide antibodies. Serodiag. Immun. Infect. Dis. 3, 111-121.

SCHIFFER, M.S., OLIVEIRA, E., GLODE, M., McCRACKEN, G.H., Jr., SARFF, L.M. & ROBBINS, J.B. (1976) A review: Relation between invasiveness and the K1 capsular polysaccharide of *Escherichia coli*. Pediatr. Res. 10, 82-87.

SCHILLER, N.L. (1988) Characterization of the susceptibility of *Pseudomonas aeruginosa* to complement-mediated killing: role of antibodies to the rough lipopolysaccharide on serum-sensitive strains. Infect. Immun. 56, 632-639.

SCHINDLER, M. & OSBORN, M.J. (1979) Interaction of divalent cations and polymyxin B with lipopolysaccharide. Biochemistry 18, 4425-4430.

SCHMEISER, T., KURRLE, E., ARNOLD, R., KRIEGER, D., HEIT, W. & HEIMPEL, H. (1988) Antimicrobial prophylaxis in neutropenic patients after bone marrow transplantation. Infection 16, 19-24.

SCHMIDT, G., JANN, B. & JANN, K. (1970) Immunochemistry of R lipopolysaccharides of *Escherichia coli*: studies on R mutants with incomplete core, derived from *E. coli* O8:K27. Eur. J. Biochem. 16, 382-392.

SCHMIDT, M.A. & JANN, K. (1982) Phospholipid substitution of capsular (K) polysaccharide antigens from *Escherichia coli* causing extra-intestinal infections. FEMS Microbiol. Lett. 14, 69-74.

SCHMIDT, R.E., HARTLAPP, J.H., NIESE, D., ILLIGER, H.J. & STROEHMANN, I. (1984) Reduction of infection frequency by intravenous gammaglobulins during intensive induction therapy for small cell carcinoma of the lung. Infection 12, 167-170.

SCHROFF, R., FOON, K., BEATTY, S., OLDHAM, R. & MORGAN, A., Jr. (1985) Human anti-murine immunoglobulin responses in patients receiving monoclonal antibody therapy. Cancer Res. 45, 879-885.

SCHUMANN, R.R., LEONG, S.R., FLAGGS, G.W., GRAY, P.W., WRIGHT, S.D., MATHISON, J.C., TOBIAS, P.S. & ULEVITCH, R.J. (1990) Structure and function of lipopolysaccharide binding protein. Science 249, 1429-1431.

SCHWARTZER, T.A., ALCID, D.V., NUMSUWAN, A.V.S. & GOCKE, D.J. (1989) Immunological specificity of human antibodies to lipopolysaccharide from the J5 rough mutant of *Escherichia coli* O111:B4. J. Infect. Dis. 159, 35-41.

SCOTT, B.B. & BARCLAY, G.R. (1987) Endotoxin-polymyxin complexes in an improved enzyme-linked immunosorbent assay for IgG antibodies in blood donar sera to Gram-negative endotoxin core glycolipids. Vox. Sang. 52, 272-280.

SCOTT, B.B. & BARCLAY, G.R. (1990) IgG antibodies to Gram-negative endotoxin in human sera. II. Opsonic activity of cross-reactive antibodies to endotoxin core for rough and smooth bacteria. Serodiag. Immunother. Infect. Dis. 4, 39-51.

SCOTT, B.B., BARCLAY, G.R., SMITH. D.G.E., McLOUGHLIN, F. & POXTON, I.R. (1990) IgG antibodies to Gram-negative endotoxin in human sera. I. Lipopolysaccharide (LPS) cross-reactivity due to antibodies to LPS core. Serodiag. Immunother. Infect. Dis. 4, 25-38.

SHENEP, J.L., BARRETT, F.F., STIDHAM, G.L., WESTENKIRCHNER, D.F. & FLYNN, P. (1985a) Endotoxin liberation during therapy for Gram-negative bacterial sepsis. Crit. Care Med. 13, 289.

SHENEP, J.L., BARTON, R.P. & MOGAN, K.A. (1985b) Role of antibiotic class in the rate of liberation of endotoxin during therapy for experimental Gram-negative bacterial sepsis. J. Infect. Dis. 151, 1012-1018.

SHENEP, J.L., FLYNN, P.M., BARRETT, F.F., STIDHAM, G.L. & WESTENKIRCHNER, D.F. (1988) Serial quantitation of endotoxemia and bacteremia during therapy for Gram-negative bacterial sepsis. J. Infect. Dis. 157, 565-568.

SHENEP, J.L. & MOGAN, K.A. (1984) Kinetics of endotoxin release during antibiotic therapy for expermental Gram-negative bacterial sepsis. J. Infect. Dis. 150, 380-388.

SHEPPARD, B.C., FRAKER, D.L. & NORTON, J.A. (1989) Prevention and treatment of endotoxin and sepsis lethality with recombinant human tumor necrosis factor. Surgery 106, 156-161.

SHERBERT, G.V. & LAKSHMI, M.S. (1973) Characterisation of *Escherichia coli* cell surface by isoelectric equilibrium analysis. Biochim. Biophys. Acta 15, 533-540.

SHIBL, A.M. (1983) Effect of antibiotics on production of enzymes and toxins by microorganisms. Rev. Infect. Dis. 5, 865-875.

SIBER, G.R., KANIA, S.A., WARREN, H.S. (1985) Cross-reactivity of rabbit antibodies to lipopolysaccharides of *Escherichia coli* J5 and other Gram-negative bacteria. J. Infect. Dis. 152, 954-964.

SILVA, A.T., APPELMELK, B.J., BUURMAN, W.A., BAYSTON, K.F. & COHEN, J. (1990a) Monoclonal antibody to endotoxin core protects mice from *Escherichia coli* sepsis by a mechanism independent of tumor necrosis factor and interleukin-6. J. Infect. Dis. 162, 454-459.

SILVA, A.T., BAYSTON, K.F. & COHEN, J. (1990b) Prophylactic and therapeutic effects of a monoclonal antibody to tumor necrosis factor-α in experimental Gram-negative shock. J. Infect. Dis. 162, 421-427.

SILVER, R.P. & VIMR, E.R. (1990) Polysialic acid capsule of *Escherichia coli* K1. In Molecular Basis of Bacterial Pathogenesis, pp 39-60. Edited by B.H. Iglewski & V.L. Clark. Academic Press Inc., San Diego.

DE SIMONE, C., DELOGU, G. & CORBETTA, G. (1988) Intravenous immunoglobulins in association with antibiotics: a therapeutic trial in septic intensive care unit patients. Crit. Care Med. 16, 23-26.

SKARNES, R.C. (1985) *In vivo* distribution and detoxification of endotoxins. In Handbook of Endotoxin. Vol 3: Cellular Biology of Endotoxin, pp 56-81. Edited by L.J. Berry. Elsevier, Amsterdam.

SMITH, H. (1977) Microbial surfaces in relation to pathogenicity. Bacteriol. Rev. 41, 475-500.

SMITH, C.C. (1990) Antibiotic usage in septicaemic shock: choice of therapy. Care Crit. Ill 6, 217-222.

SORI, A.J., RUSH, B.F., Jr., LYSZ, T.W., SMITH, S. & MACHIEDO, G.W. (1988) The gut as source of sepsis after hemorrhagic shock. Am. J. Surg. 155, 187-191.

SPEAR, G.T. & TEODORESCU, M. (1984) Enhancement of human MLR by very low concentrations of lipopolysaccharide and blocking of this enhancement by polymyxin B. J. Immunol. Methods 73, 321-327.

SPEERT, D.P., FARMER, S.W., CAMPBELL, M.E., MUSSER, J.M., SELANDER, R.K. & KUO, S. (1990) Conversion of *Pseudomonas aeruginosa* to the phenotype characteristic of strains from patients with cystic fibrosis. J. Clin. Microbiol. 28, 188-194.

SPERBER, S.J. & SCHLEUPNER, C.J. (1987) Salmonellosis during infection with human immunodeficiency virus. Rev. Infect. Dis. 9, 925-934.

STEADMAN, R., TOPLEY, N., JENNER, D.E., DAVIES, M. & WILLIAMS, J.D. (1988) Type 1 frimbriale *Escherichia coli* stimulates a unique pattern of degranulation by human polymorphonuclear leukocytes. Infect. Immun. 56, 815-822.

STEEN, H.B. & BOYE, E. (1980) Bacterial growth studied by flow cytometry. Cytometry 1, 32-35.

STERKENBURG, A., VLEGELS, E. & WOUTERS, J.T.M. (1984) Influence of nutrient limitation and growth rate on the outer membrane proteins of *Klebsiella aerogenes* NCTC 418. J. Gen. Microbiol. 130, 2347-2355.

STEVENS, P., HUANG, S.N.H., WELCH, W.D. & YOUNG, L.S. (1978) Restricted complement activation by *Escherichia coli* with the K-1 capsular serotype: a possible role in pathogenicity. J. Immunol. 121, 2174-2180.

STOKES, D.C., SHENEP, J.L., FISHMAN, M., HILDNER, W.K., BYSANI, G.K. & RUFUS, K. (1989) Polymyxin B prevents lipopolysaccharide-induced release of tumor necrosis factor-α from alveolar macrophages. J. Infect. Dis. 160, 52-57.

STRAUS, D.C. (1987) Production of an extracellular toxic complex by various strains of *Klebsiella pneumoniae*. Infect. Immun. 55, 44-48.

STRAUS, D.C., WOODS, D.E., LONON, M. & GARNER, C.W. (1988) The importance of extracellular antigens in *Pseudomonas cepacia* infections. J. Med. Micro. 26, 269-280.

STURK, A. & TEN CATE, J.W. (1985) Endotoxin testing revisited. Eur. J. Clin. Microbiol. 4, 382-385.

SUERBAUM, S., LEYING, H., KROLL, H.-P., GMEINER, J. & OPFERKUCH, W. (1987) Influence of β-Lactam antibiotics and ciprofloxacin on cell envelope of *Escherichia coli*. Antimicrob. Agents Chemother. 31, 1106-1110.

SUFFREDINI, A.F., FROMM, R.E., PARKER, M.M., BRENNER, M., KOVACS, J.A., WESLEY, R.A. & PARRILLO, J.E. (1989) The cardiovascular response of normal humans to the administration of endotoxin. New Engl. J. Med. 321, 280-287.

SUTHERLAND, I.W. (1977) Bacterial exopolysaccharides - their nature and production. In Surface carbohydrates of the prokaryotic cell, pp 27-96. Edited by I.W. Sutherland. Academic Press, London.

SUTHERLAND, I.W. (1985) Biosynthesis and composition of Gram-negative bacterial extracellular and wall exopolysaccharides. Ann. Rev. Microbiol. 39, 243-270.

SUTHERLAND, I.W. & WILKINSON, J.F. (1965) Depolymerases for bacterial exopolysaccharides obtained from phage-infected bacteria. J. Gen. Microbiol. 39, 373-383.

TACKEN, A., RIETSCHEL, E.TH. & BRADE, H. (1986) Methylation analysis of the heptose/3-deoxy-D-manno-2-octulosonic acid region (inner core) of the lipopolysaccharide from *Salmonella minnesota* rough mutants. Carbohydr. Res. 149, 279-291.

TAMAKUMA, S., ROJAS-CORONA, R., CUEVAS, P. & FINE, J. (1971) Demonstration of a lethal endotoxemia of intestinal origin in refractory non-septic shock. Ann. Surg. 173, 219-224.

TAMURA, H., TANAKA, S., OBAYASHI, T., YOSHIDA, M. & KAWAI, T. (1991) A new sensitive method for determining endotoxin in whole blood. Clin. Chim. Acta 200, 35-42.

TANAMOTO, K.-I., ZAHRINGER, U., McKENZIE, G.R., GALANOS, C., RIETSCHEL, E.T., LÜDERITZ, O., KUSUMOTO, S. & SHIBA, T. (1984) Biological activities of synthetic lipid A analogs: pyrogenicity, lethal toxicity anticomplement activity, and induction of gelation of *Limulus* amoeboaye lysate. Infect. Immun. 44, 421-426.

TAYLOR, P.W. (1983) Bactericidal and bacteriolytic activity of serum against Gram-negative bacteria. Microbiol. Rev. 47, 46-83.

TAYLOR, P.W., GAUNT, H. & UNGER, F.M. (1981a) Effect of subinhibitory concentrations of mecillinam on the serum susceptibility of *Escherichia coli* strains. Antimicrob. Agents Chemother. 19, 786-788.

TAYLOR, P.W., KROLL, H.-P. & TOMLINSON, S. (1982) Effect of subinhibitory concentrations of mecillinam on expression of *Escherichia coli* surface components associated with serum resistance. Drugs Exp. Clin. Res. 8, 625-631.

TAYLOR, P.W., MESSNER, P. & PARTON, R. (1981b) Effect of the growth environment on cell-envelope components of *Escherichia coli* in relation to sensitivity to human serum. J. Med. Micro. 14, 9-19.

TAYLOR, P.W. & ROBINSON, M.K. (1980) Determinants that increase the serum resistance of *Escherichia coli*. Infect. Immun. 29, 278-280.

TENG, N.N.H., KAPLAN, H.S., HEBERT, J.M., MOORE, C., DOUGLAS, H., WUNDERLICH, A. & BRAUDE, A.I. (1985) Protection against Gram-negative bacteremia and endotoxemia with human monoclonal IgM antibodies. Proc. Natl. Acad. Sci. USA. 82, 1790-1794.

TERRY, J.M., PIRIA, S.S. & MATTINGLY, S.J. (1991) Environmental conditions which influence mucoid conversion in *Pseudomonas aeruginosa* PAO1. Infect. Immun. 59, 471-477.

TEUBER, M. (1974) Action of polymyxin B on bacterial membranes. III. Differential inhibition of cellular functions in *Salmonella typhimurium*. Arch. Mikrobiol. 100, 131-144.

THOMAS, L.L., STURK, A., BULLER, H.R., TEN CATE, J.W., SPIKER, R.E. & TEN CATE, H. (1984) Comparative investigation of a quantitative chromogenic endotoxin assay and blood cultures. Am. J. Clin. Pathol. 82, 203-206.

THORENS, B., MERMOD, J.-J. & VASSALLI, P. (1987) Phagocytosis and inflammatory stimuli induce GM-CSF mRNA in macrophages through post-transcriptional regulation. Cell, 48, 671-679.

TOBIAS, P.S., SOLDAU, K. & ULEVITCH, R.J. (1986) Isolation of a lipopolysaccharide-binding acute phase reactant from rabbit serum. J. Exp. Med. 164, 777-793.

TOBIAS, P.S., SOLDAU, K. & ULEVITCH, R.J. (1989) Identification of a lipid A binding site in the acute phase reactant lipopolysaccharide binding protein. J. Biol. Chem. 264, 10867-10871.

TOMAS, J.M., BENEDI, V.J., CIURANA, B.C. & JOFRE, J. (1986) Role of capsule and O antigen in resistance of *Klebsiella pneumoniae* to serum bactericidal activity. Infect. Immun. 54, 85-89.

TOMAS, J.M., CIURANA, B., BENEDI, V.J. & JUAREZ, A. (1988) Role of lipopolysaccharide and complement in susceptibility of *Escherichia coli* and *Salmonella typhimurium* to non-immune serum. J. Gen. Microbiol. 134, 1009-1016.

TOWBIN, H., STAEHELIN, T. & GORDON, J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA 76, 4350-4354.

TRACEY, K.J., FONG, Y., HESSE, D.G., MANOGUE, K.R., LEE, A.T., KUO, G.C., LOWRY, S.F. & CERAMI, A. (1987) Anti-cachectin/TNF monoclonal antibodies prevent septic shock during lethal bacteraemia. Nature 330, 662-664.

TRAUTMANN, M. & HAHN, H. (1985) Antiserum against *Escherichia coli* J5: a re-evaluation of its *in vitro* and *in vivo* activity against heterologous bacteria. Infection 13, 140-145.

TRIGER, D.R., BOYER, T.D. & LEVIN, J. (1978) Portal and systemic bacteraemia and endotoxemia in liver disease. Gut 19, 935-939.

TROY, F.A., FREEMAN, F.E. & HEATH, E.C. (1971) The biosynthesis of the capsular polysaccharide in Aerobacter aerogenes. J. Biol. Chem. 246, 118-133.

TSAI, C.-M. & FRASCH, C.E. (1982) A sensitive silver stain for detecting lipopolysaccharide in polyacrylamide gels. Anal. Biochem. 119, 115-119.

TSUCHIDO, T., KATSUI, N., TAKEUCHI, A., TAKANO, M. & SHIBASAKI, I. (1985) Destruction of the outer membrane permeability barrier of *Escherichia coli* by heat treatment. Appl. Environ. Microbiol. 50, 298-303.

TUOMANEN, E., COZENS, R.M., TOSCH, W., ZAK, O. & TOMASZA, A. (1986) The rate of killing of *Escherichia coli* by β-lactam antibiotics is strictly proportional to the rate of bacterial growth. J. Gen. Microbiol. 132, 1297-1304.

TYNDALL, R.L., HAND, R.E., Jr., MANN, R.C., EVANS, C. & JERNIGAN, R. (1985) Application of flow cytometry to detection and characterization of *Legionella* spp. Appl. Environ. Microbiol. 49, 852-857.

UHLIN, B.E., BAGA, M., GORANSSON, M., LINDBERG, F.P., LUND, B., NORGREN, M. & NORMARK, S. (1985) Genes determining adhesin formation in uropathogenic *Escherichia coli*. Curr. Top. Microbiol. Immunol. 118, 163-178.

ULEVITCH, R.J. & JOHNSTON, A.R. (1978) The modification of biophysical and endotoxic properties of bacterial lipopolysaccharides by serum. J. Clin. Invest. 62, 1313-1324.

ULEVITCH, R.J., JOHNSTON, A.R. & WEINSTEIN, D.B. (1979) New function for high density lipoproteins. Their participation in intravascular reactions of bacterial lipopolysaccharides. J. Clin. Invest. 64, 1516-1524.

ULEVITCH, R.J., JOHNSTON, A.R. & WEINSTEIN, D.B. (1981) New functions for high density lipoproteins. Isolation and characterization of a bacterial lipopolysaccharide-high density lipoprotein complex formed in rabbit plasma. J. Clin. Invest. 67, 827-837.

ULEVITCH, R.J. & TOBIAS, P.S. (1988) Interactions of bacterial lipopolysaccharides with serum proteins. In Bacterial Endotoxins: Pathophysiological Effects, Clinical Significance, and Pharmacological Control. Progr. Clin. Biol. Res., 272, p309. Edited by J. Levin, H.R. Büller, J.W. Cate, S.J.H. Van Deventer & A. Sturk. Alan R. Liss, New York.

VAARA, M. & HIROSHI, H. (1984) Molecular organization of bacterial outer membrane. In Handbook of endotoxin. Vol.1. Chemistry of endotoxin, pp 1-45. Edited by E.Th. Rietschel, Elsevier, Amsterdam.

VAARA, M. & NIKAIDO, H. (1984) Molecular organization of bacterial outer membrane. In Handbook of endotoxin. Vol. 1. Chemistry of endotoxin, pp 1-45. Edited by E.Th. Rietschel. Elsevier, Amsterdam.

VANN, W.F., SCHMIDT, M.A., JANN, B. & JANN, K. (1981) The structure of capsular polysaccharide (K5 antigen) of urinary tract-infective *Escherichia coli* O10:K5:H4. A polymer similar to desulfo-heparin. Eur. J. Biochem. 116, 359-364.

VASIL, M.L., BERKA, R.M., GRAY, G.L. & PAVLOUSKI, O.R. (1985) Biochemical and genetic studies of iron-regulated (exotoxin A) and phosphate-regulated (hemolysin phospholipase C) virulence factors of *Pseudomonas aeruginosa*. Antibiot. Chemother. 36, 23-39.

VERINGA, E., BOX, A., ROZENBERG-ARSKA, M. & VERHOEF, J. (1988) Monobactam antibiotics in sub-inhibitory concentrations enhance opsonophagocytosis and serum bacteriolysis in certain *Escherichia coli* strains. Drugs Exp. Clin. Res. XIV, 1-8.

VERMEULEN, C., CROSS, A., BYRNE, W.R. & ZOLLINGER, W. (1988) Quantitative relationship between capsular content and killing of K1-encapsulated *Escherichia coli*. Infect. Immun. 56, 2723-2730.

VAN VERSEVELD, H.W., BAKKER, P., VAN DER WOUDE, T., TERLETH, C. & DE GRAAF, F.K. (1985) Production of fimbrial adhesins K99 and K41 by enterotoxigenic *Escherichia coli* as a function of growth-rate domain. Infect. Immun. 49, 159-163.

VUKAJLOVICH, S.W. (1986) Antibody-independent activation of the classical pathway of human serum complement by lipid A is restricted to Re-chemotype lipopolysaccharide and purified lipid A. Infect. Immun. 53, 480-485.

VUOPIO-VARKILA, J., NURMINEN, M., PYHALA, L. & MAKELA, P.H. (1988) Lipopolysaccharide-induced non-specific resistance to systemic *Escherichia coli* infection in mice. J. Med. Micro. 25, 197-203.

WAAGE, A., BRANDTZAEG, P., HALSTENSEN, A., KIERULF, P. & ESPEVIK, T. (1989) The complex pattern of cytokines in serum from patients with meningococcal septic shock. Association between interleukin 6, interleukin 1, and fatal outcome. J. Exp. Med. 169, 333-338.

WARD, D.C., MICHALEK, S.M. & McGHEE, J.R. (1988) Monoclonal antibodies to salmonella lipopolysaccharide: functional analysis of anti-lipid A antibodies. Clin. Exp. Immunol. 72, 157-163.

WARDLE, E.N. (1979) Shock-bacteraemic and endotoxic shock. Br. J. Hosp. Med. 21, 223-231.

WARREN, H.S., GLENNON, M., de DECKKER, F.A. & TELLO, D. (1991) Role of normal serum in the binding of lipopolysaccharide to IgG fractions from rabbit antisera to *Escherichia coli* J5 and other Gram-negative bacteria. J. Infect. Dis. 163, 1256-1266.

WARREN, H.S., KNIGHTS, C.V. & SIBER, G.R. (1986) Neutralization and lipoprotein binding of lipopolysaccharides in tolerant rabbit serum. J. Infect. Dis. 154, 784-791.

WEINBERG, E.D. (1978) Iron and infection. Microbiol. Rev. 42, 45-66.

WEINBERG, E.D. (1984) Iron withholding: a defense against infection and neoplasia. Physiol. Rev. 64, 65-102.

WEINBERG, J.R., WRIGHT, D.J.M. & GUZ, A. (1988) Interleukin-1 and tumour necrosis factor cause hypotension in the conscious rabbit. Clin. Sci. 75, 251-255.

WEINSTEIN, M.P., RELLER, L.B., MURPHY, J.R. & LICHTENSTEIN, K.A. (1983) The clinical significance of positive blood cultures: a comprehensive analysis of 500 episodes of bacteremia and fungemia in adults. 1. Laboratory and epidemiologic observations. Rev. Infect. Dis. 5, 35-53.

WEISS, A.A. & HEWLETT, E.L. (1986) Virulence factors of *Bordetella pertussis*. Annu. Rev. Microbiol. 40, 661-686.

WEISS, A.A., HEWLETT, E.L., MYERS, G.A. & FALKOW, S. (1983) Tn5-induced mutations affecting virulence factors of *Bordetella pertussis*. Infect. Immun. 42, 33-41.

WEISS, J., HUTZLER, M. & KAO, L. (1986) Environmental modulation of lipopolysaccharide chain length alters the sensitivity of *Escherichia coli* to the neutrophil bactericidal/permeability-increasing protein. Infect. Immun. 51, 594-599.

WELLS, M. & GAFFIN, S.L. (1987) Antipseudomonas activity of anti-lipopolysaccharide hyperimmune equine plasma. Clin. Exp. Immunol. 68, 86-92.

WESSELS, B.C., WELLS, M.T., GAFFIN, S.L., BROCK-UTNE, J.G., GATHIRAM, P. & HINSHAW, L.B. (1988) Plasma endotoxin concentration in healthy primates and during *E. coli*-induced shock. Crit. Care Med. 16, 601-605.

WEST, T.E. & APICELLA, M.A. (1985) Microbial factors in the pathogenesis of sepsis. In Septic Shock, pp 27-40. Edited by R.K. Root & M.A. Sande. Churchill Livingstone, New York.

WESTPHAL, O., JANN, K. & HIMMELSPACH, K. (1983) Chemistry and immunochemistry of bacterial lipopolysaccharides as cell wall antigens and endotoxins. Prog. Allerg. 33, 9-39.

WESTPHAL, O. & LÜDERITZ. O. (1954) Chemische Erforschung von Lipopolysacchariden gramnegativer Bakterien. Angew. Chem. 66, 407-417.

WHITE, B. (1938) Antibodies to pneumococcus. In The Biology of pneumococcus, pp 355-426. Edited by B. White, E.S. Robinson & L.A. Barnes. Harvard University Press, Cambridge.

WHITFIELD, C., VIMR, E.R., COSTERTON, W. & TROY, F.A. (1984) Protein synthesis is required for *in vivo* activation of polysialic acid capsule synthesis in *Escherichia coli* K1. J. Bacteriol. 159, 321-328.

WICKEN, A.J. & KNOX, K.W. (1980) Bacterial cell surface amphiphiles. Biochim. Biophys. Acta 604, 1-26.

WIESNER, R.H., HERMANS, P.E., RAKELA, J., WASSHINGTON, J.A., PERKINS, J.D., DICECCO, S. & CROM, R. (1988) Selective bowel decontamination to decrease Gram-negative aerobic bacterial and *Candida* colonization and prevent infection after orthooptic liver transplantation. Transplantation 45, 570-574.

WILKINSON, S.G. (1977) Composition and structure of bacterial lipopolysaccharides. In Surface carbohydrates of the prokaryotic cell. pp 97-175. Edited by I.W. Sutherland. Academic Press, New York.

WILKINSON. S.G. (1983) Composition and structure of lipopolysaccharides from *Pseudomonas aeruginosa*. Rev. Infect. Dis. 5, Suppl. 5, S941-S949.

WILLIAMS, P. (1987) Sub-MICs of cefuroxime and ciprofloxacin influence interaction of complement and immunoglobulins with *Klebsiella pneumoniae*. Antimicrob. Agents Chemother. 31, 758-762.

WILLIAMS, P. (1988) Role of the cell envelope in bacterial adaptation to growth *in vivo* in infections. Biochimie 70, 987-1011.

WILLIAMS, P., LAMBERT, P.A. & BROWN, M.R.W. (1988) Penetration of immunoglobulins through the *Klebsiella* capsule and their effect on cell-surface hydrophobicity. J. Med. Micro. 26, 29-35.

WILLIAMS, P., LAMBERT, P.A., BROWN, M.R.W. & JONES, R.J. (1983) The role of the O and K antigens in determining the resistance of *Klebsiella aerogenes* to serum killing and phagocytosis. J. Gen. Microbiol. 129, 2181-2191.

WILLIAMS, P., LAMBERT, P.A., HAIGH, C.G. & BROWN, M.R.W. (1986) The influence of the O and K antigens of *Klebsiella aerogenes* on surface hydrophobicity and susceptibility to phagocytosis and antimicrobial agents. J. Med. Microbiol. 21, 125-132.

WILLIAMS, P. & TOMAS, J.M. (1990) The pathogenicity of *Klebsiella pneumoniae*. Rev. Med. Micro. 1, 196-204.

WILSON, F. (1984) Surgical intensive care units. In Major Issues in Critical Care Medicine. pp 17-33. Edited by J.E. Parrillo & S.M. Ayres. Williams & Wilkins, Baltimore.

WINCHURCH, R.A., THUPARI, J.N. & MUNSTER, M.M. (1987) Endotoxaemia in burn patients: Levels of circulating endotoxins are related to burn size. Surgery 102, 808-812.

WOLF, S.M. (1982) The treatment of Gram-negative bacteraemia and shock. N. Eng. J. Med. 30, 1267-1268.

WOLFSON, J.S. & HOOPER, D.C. (1985) The fluoroquinolones: structures, mechanisms of action and resistance, and spectra of activity *in vitro*. Antimicrob. Agents Chemother. 28, 581-586.

WOLLENWEBER, H.-W., BROADY, K.W., LÜDERITZ, O. & RIETSCHEL, E.T. (1982) The chemical structure of lipid A. Demonstration of amide-linked 3-acyloxyacyl residues in *Salmonella minnesota* Re lipopolysaccharide. Eur. J. Biochem. 124, 191-198.

WOOD, M.J. (1990) Therapy: the clinician's view. J. Antimicrob. Chemother. 25, Suppl. C, 99-106.

WRIGHT, D.G. & GALLIN, J.I. (1979) Secretory responses of human neutrophils: exocytosis of specific (secondary) granules by human neutrophils during adherence *in vitro* and during exudation *in vivo*. J. Immunol. 123, 285-294.

WRIGHT, S.D., RAMOS, R.A., TOBIAS, P.S., ULEVITCH, R.J. & MATHISON, J.C. (1990) CD14, a receptor for complexes of lipopolysaccharide (LPS) and LPS binding protein. Science 249, 1431-1433.

YOLKEN, R.H. (1982) Enzyme immunoassays for the detection of infectious diseases in body fluids: current limitations and future prospects. Rev. Infect. Dis. 4, 35-68.

YOLKEN, R.H., LEISTER, F.J., WHITCOMB, L.S. & SANTOSHAM, M. (1983) Enzyme immunoassays for the detection of bacterial antigens utilizing biotin-labelled antibody and peroxidase biotin-avidin complex. J. Immunol. Methods. 56, 319-327.

YOUNG, L.S. (1972) Human immunity to *Pseudomonas aeruginosa*. II. Relationship between heat-stable opsonins and type-specific lipopolysaccharides. J. Infect. Dis. 126, 277-287.

YOUNG, L.S. (1985) Treatment of infections due to Gram-negative bacilli: a perspective of past, present, and future. Rev. Infect. Dis. 7, Suppl. 4, S572-S578.

YOUNG, L.S., GASCON, R., ALAM, S. & BERMUDEZ, L.E.M. (1989) Monoclonal antibodies for treatment of Gram-negative infections. Rev. Infect. Dis. 11, (Suppl. 7), S1564-S1571.

YOUNG, L.S., MARTIN, W.J., MEYER, R.D., WEINSTEIN, R.J. & ANDERSON, E.T. (1977) Gram-negative rod bacteremia: microbiologic, immunologic, and therapeutic considerations. Ann. Intern. Med. 86, 456-471.

YOUNG, L.S., STEVENS, P., & INGRAM, J. (1975) Functional role of antibody against "core" glycolipid of Enterobacteriaceae. J. Clin. Invest. 56, 850-861.

YOUNG, S.E.J. (1982) Bacteraemia 1975-1980: a survey of cases reported to the PHLS Communicable Disease Surveillance Centre. J. Infect. Dis. 5, 19-26.

ZIEGLER, E.J. (1988) Protective antibody to endotoxin core: the emperor's new clothes? J. Infect. Dis. 158, 286-290.

ZIEGLER, E.J., DOUGLAS, H. & BRAUDE, A.I. (1973a) Human antiserum for prevention of the local Shwartzman reaction and death from bacterial lipopolysaccharides. J. Clin. Invest. 52, 3236-3238.

ZIEGLER, E.J., DOUGLAS, H. SHERMAN, J.E., DAVIS, C.E. & BRAUDE, A.I. (1973b) Treatment of *E. coli* and *Klebsiella* bacteremia in agranulocytic animals with antiserum to a UDP-Gal epimerase defecient mutant. J. Immunol. 111, 433-438.

ZIEGLER, E.J., FISHER, C.J., SPRUNG, C.L., STRAUBE, R.C., SADOFF, J.C., FOULKE, G.E., WORTEL, C.H., FINK, M.P., DELLINGER, R.P., TENG, N.N.H., ALLEN, I.E., BERGER, H.J., KNATTERUD, G.L., LOBUGLIO, A.F., SMITH, C.R., and the HA-1A Sepsis Study Group (1991) Treatment of Gram-negative bacteremia and septic shock with HA-1A human monoclonal antibody against endotoxin. N. Engl. J. Med. 324, 429-436.

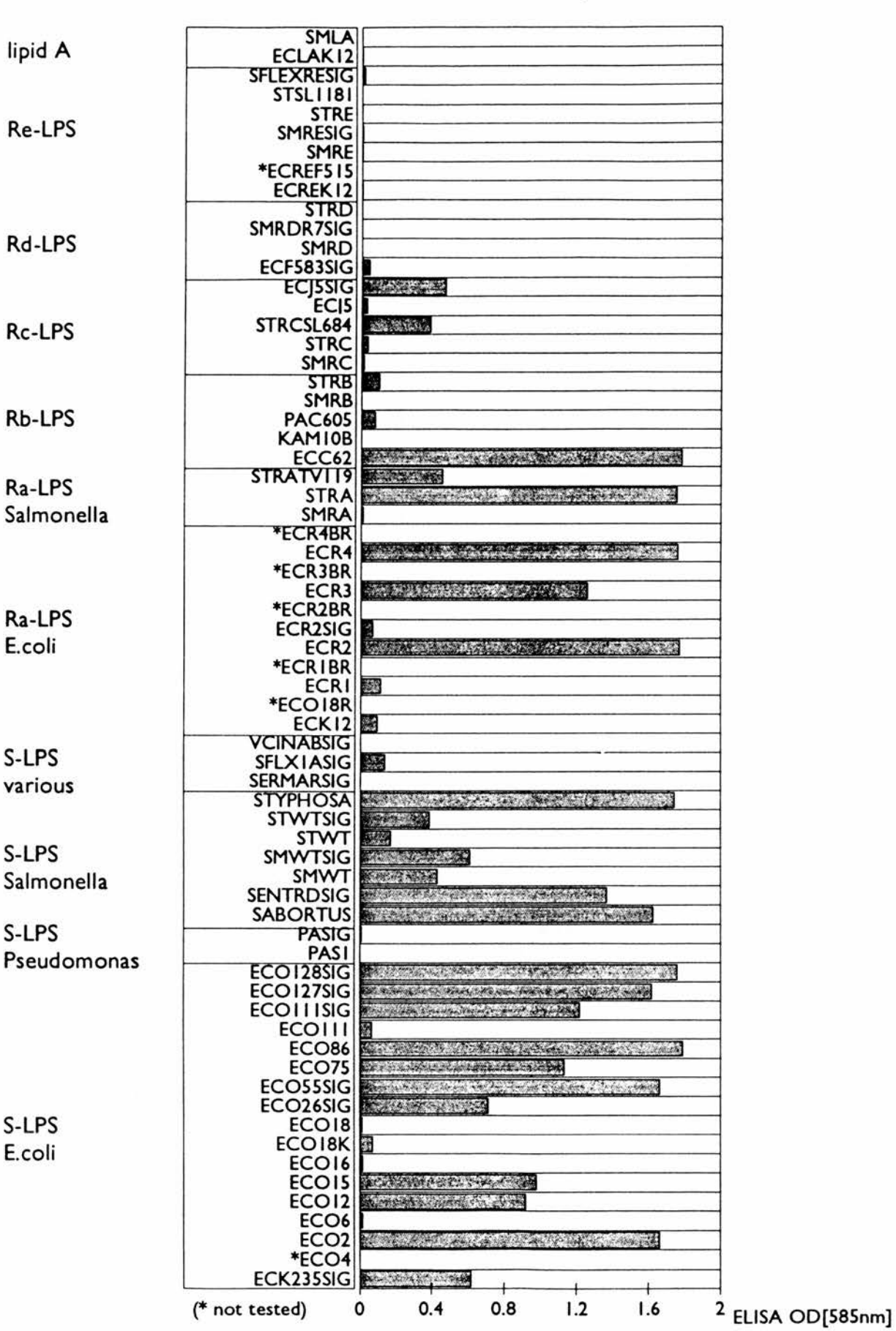
ZIEGLER, E.J., McCUTCHAN, J.A., FIERER, J., GLAUSER, M.P., SADOFF, J.C., DOUGLAS, H. & BRAUDE, A.I. (1982) Treatment of Gram-negative bacteremia and shock with human antiserum to a mutant *Escherichia coli*. N. Engl. J. Med. 307, 1225-1230.

ZIEGLER, E.J. & TENG, N.N.H. (1986) Evaluation of a human monoclonal antibody to lipid A. Riv. Immunol. Immunofarmacol. 6, 139-141.

ZORZOPULOS, J., DE LONG, S., CHAPMAN. V. & KOZLOFF, M. (1989) Evidence for a net-like organization of lipopolysaccharide particles in the *Escherichia coli* outer membrane. FEMS Microbiol. Lett. 61, 23-26.

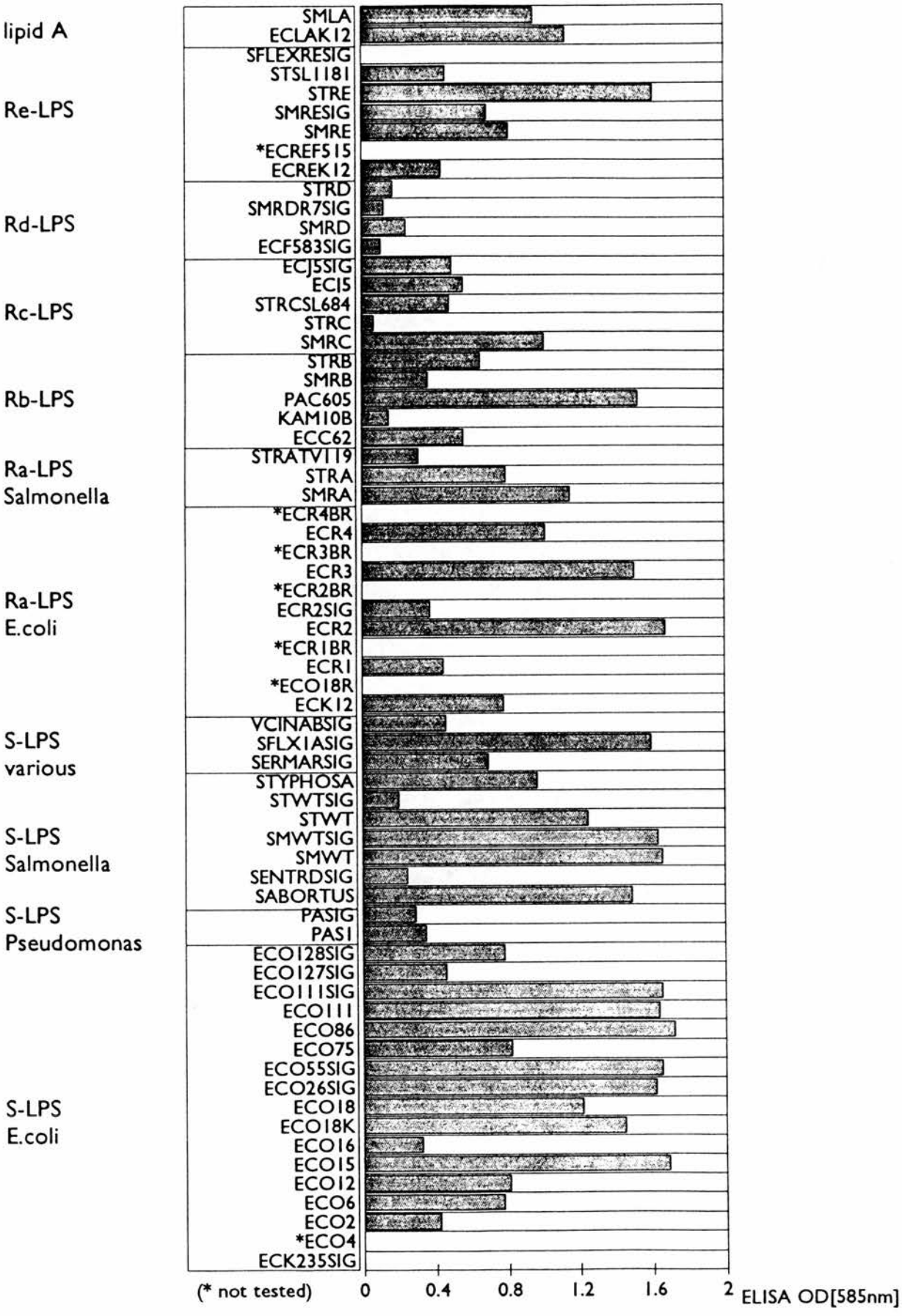
ZWEERINK, H.J., GAMMON, M.C., HUTCHISON, C.F., JACKSON, J.J., PIER, G.B., PUCKETT, J.M., SEWELL, T.J. & SIGAL, N.H. (1988) X-linked immunodeficient mice as a model for testing the protective efficacy of monoclonal antibodies against *Pseudomonas aeruginosa*. Infect. Immun. 56, 1209-1214.

APPENDIX

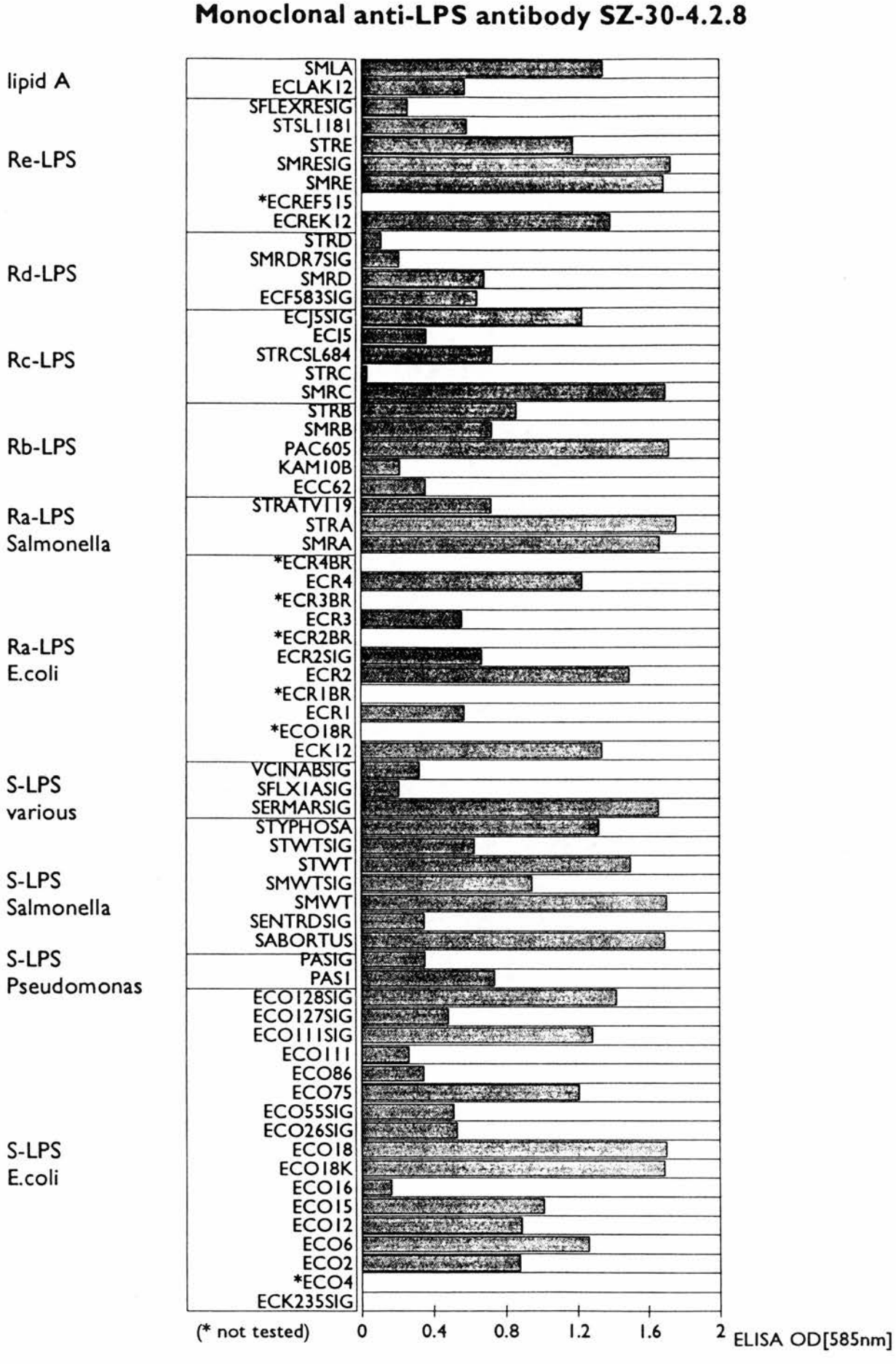


MAb specificity (maximum-binding) screening ELISA

Monoclonal anti-LPS antibody SZ-27-193.3

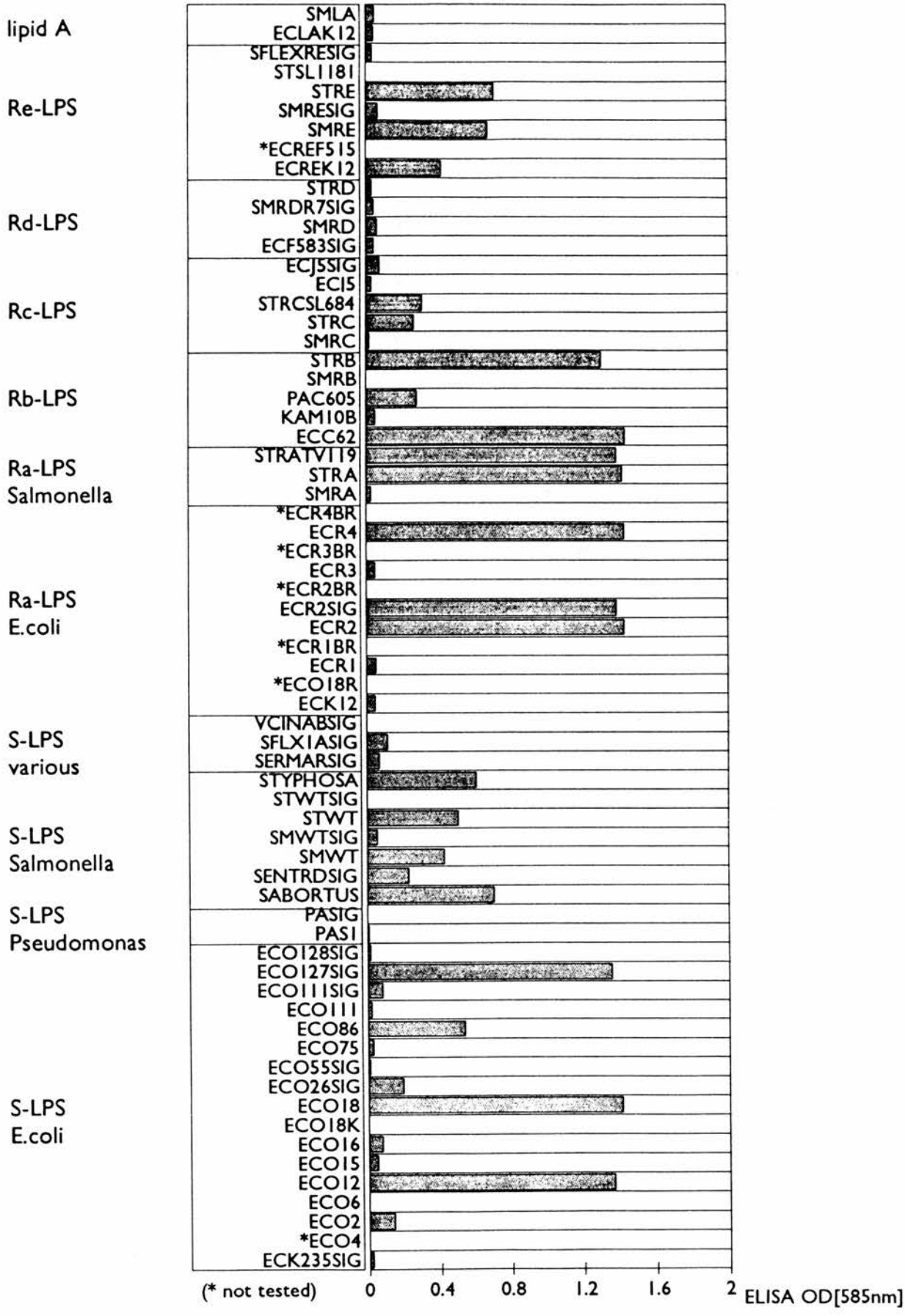


MAb specificity (maximum-binding) screening ELISA



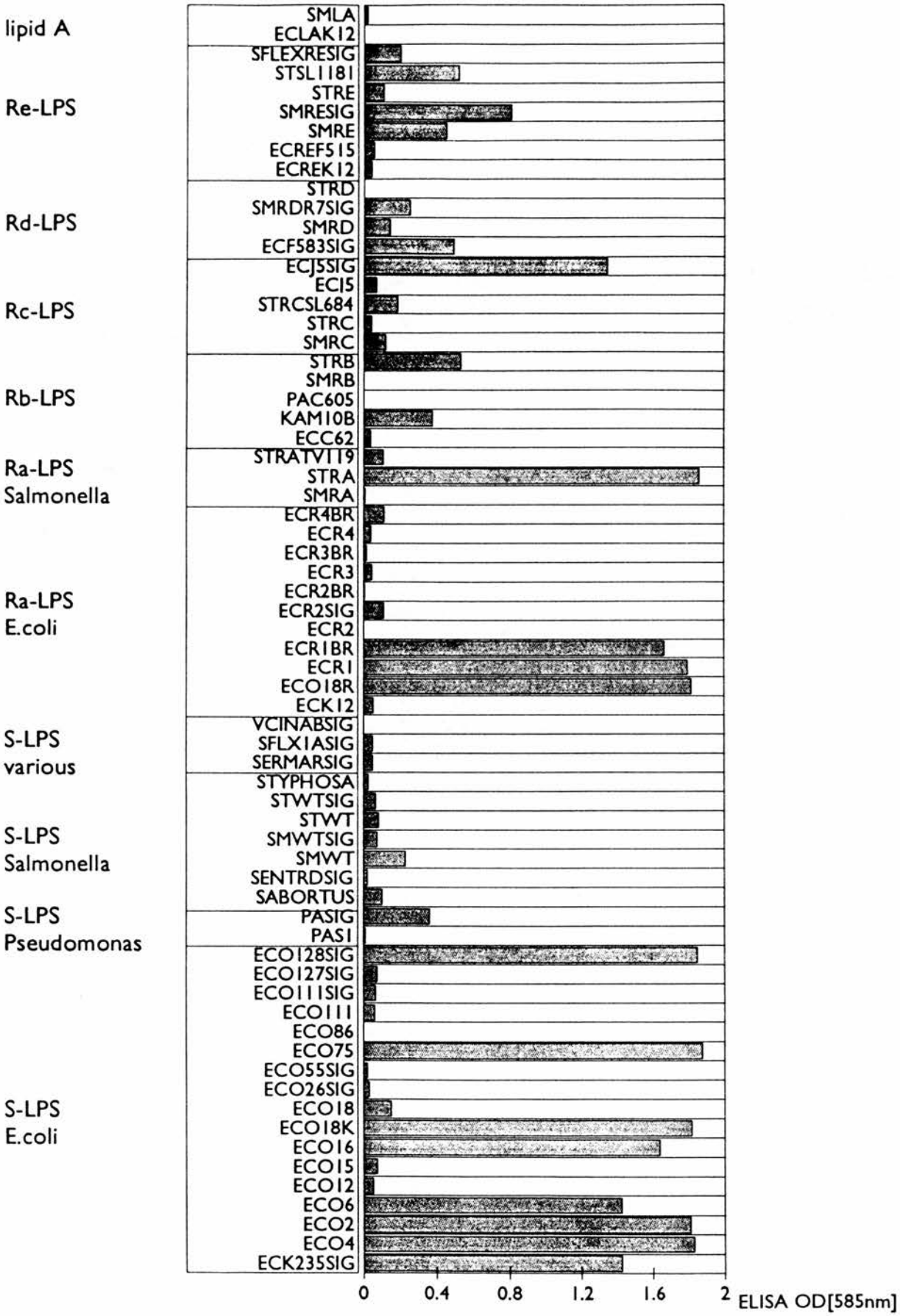
MAb specificity (maximum-binding) screening ELISA

Monoclonal anti-LPS antibody SZ-40-18.7.1



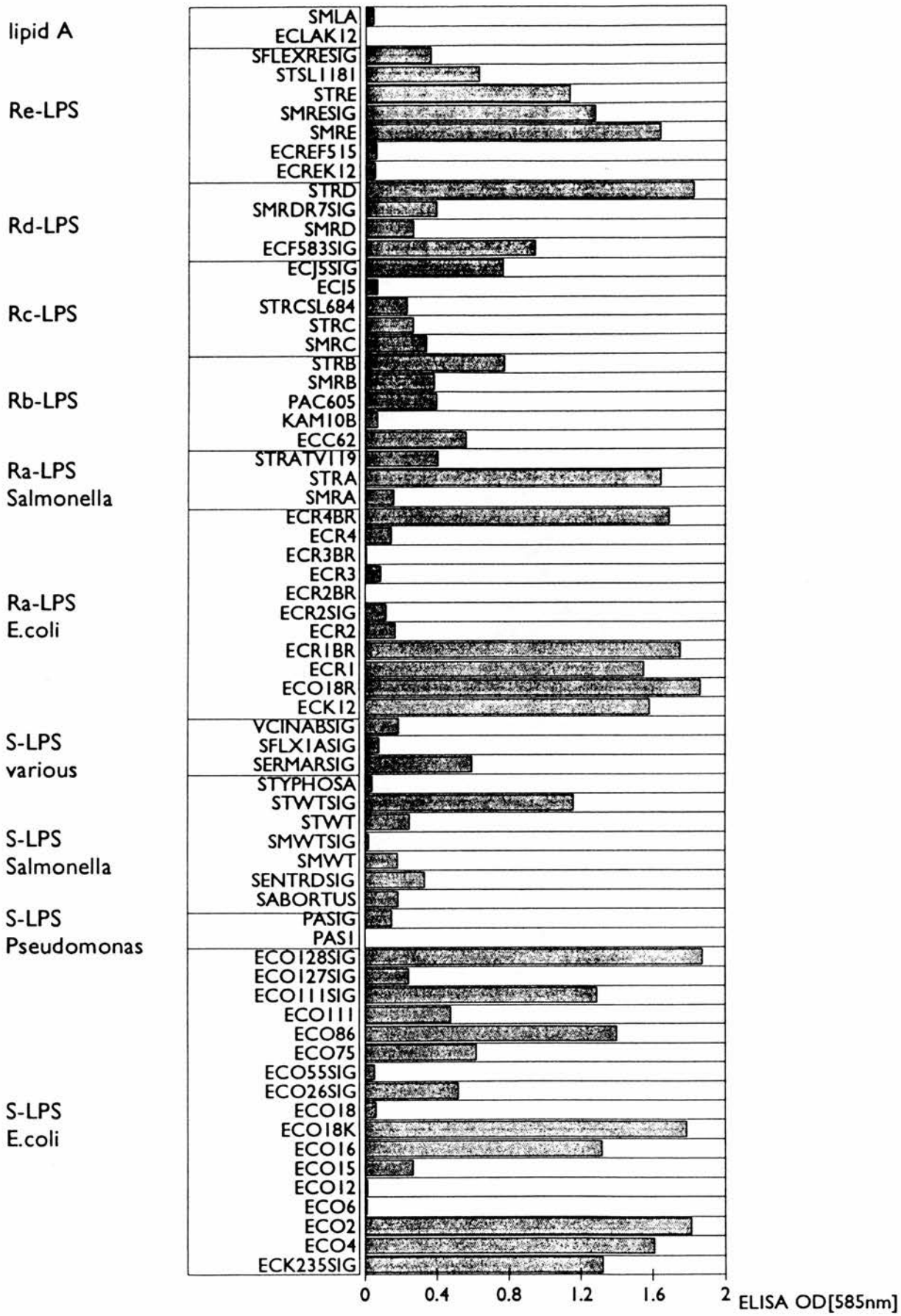
MAb specificity (maximum-binding) screening ELISA

Monoclonal anti-LPS antibody SZ-43-3.4.8

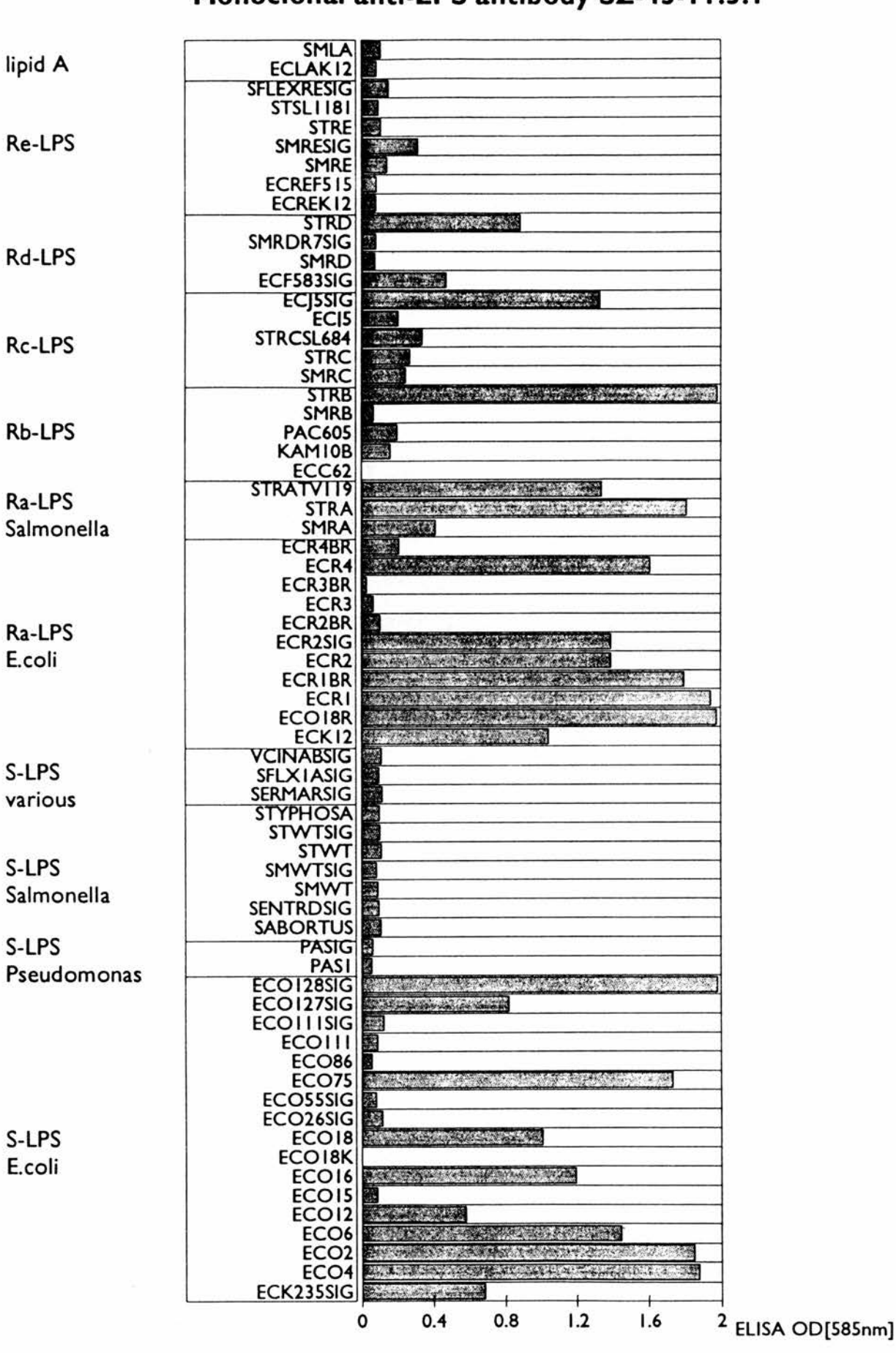


MAb specificity (maximum-binding) screening ELISA

Monoclonal anti-LPS antibody SZ-43-5.1.4

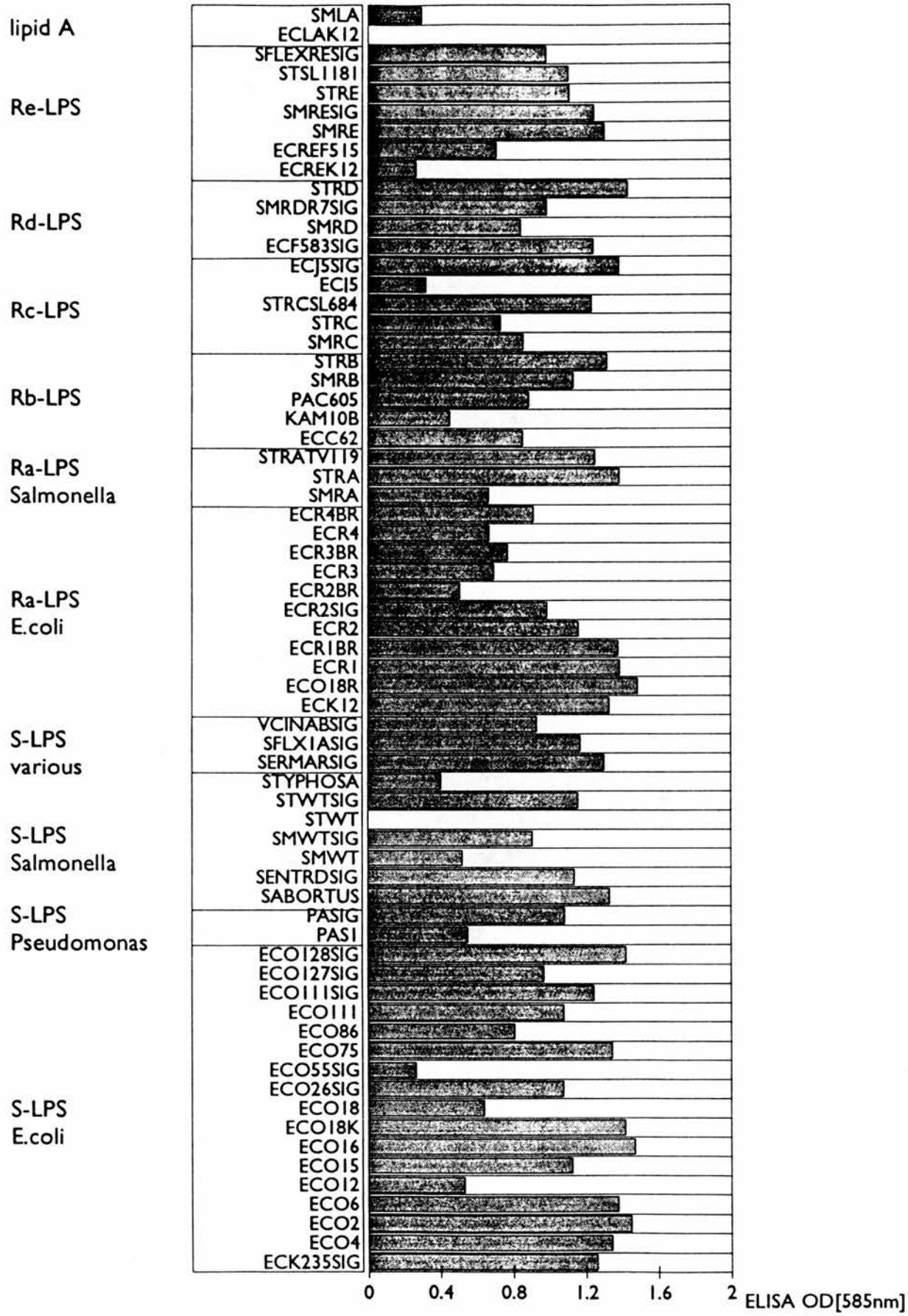


MAb specificity (maximum-binding) screening ELISA

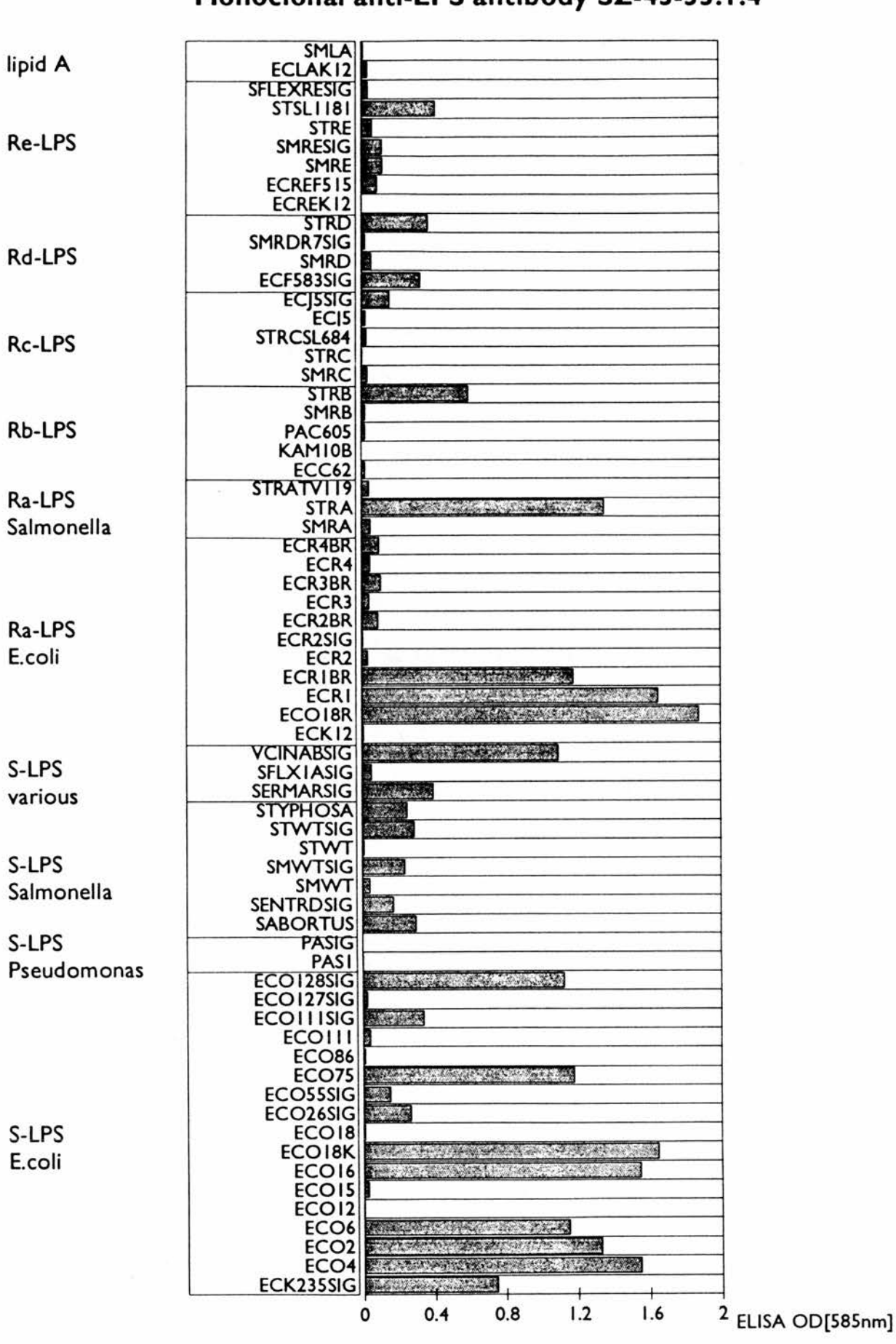


MAb specificity (maximum-binding) screening ELISA

Monoclonal anti-LPS antibody SZ-43-27.11.2

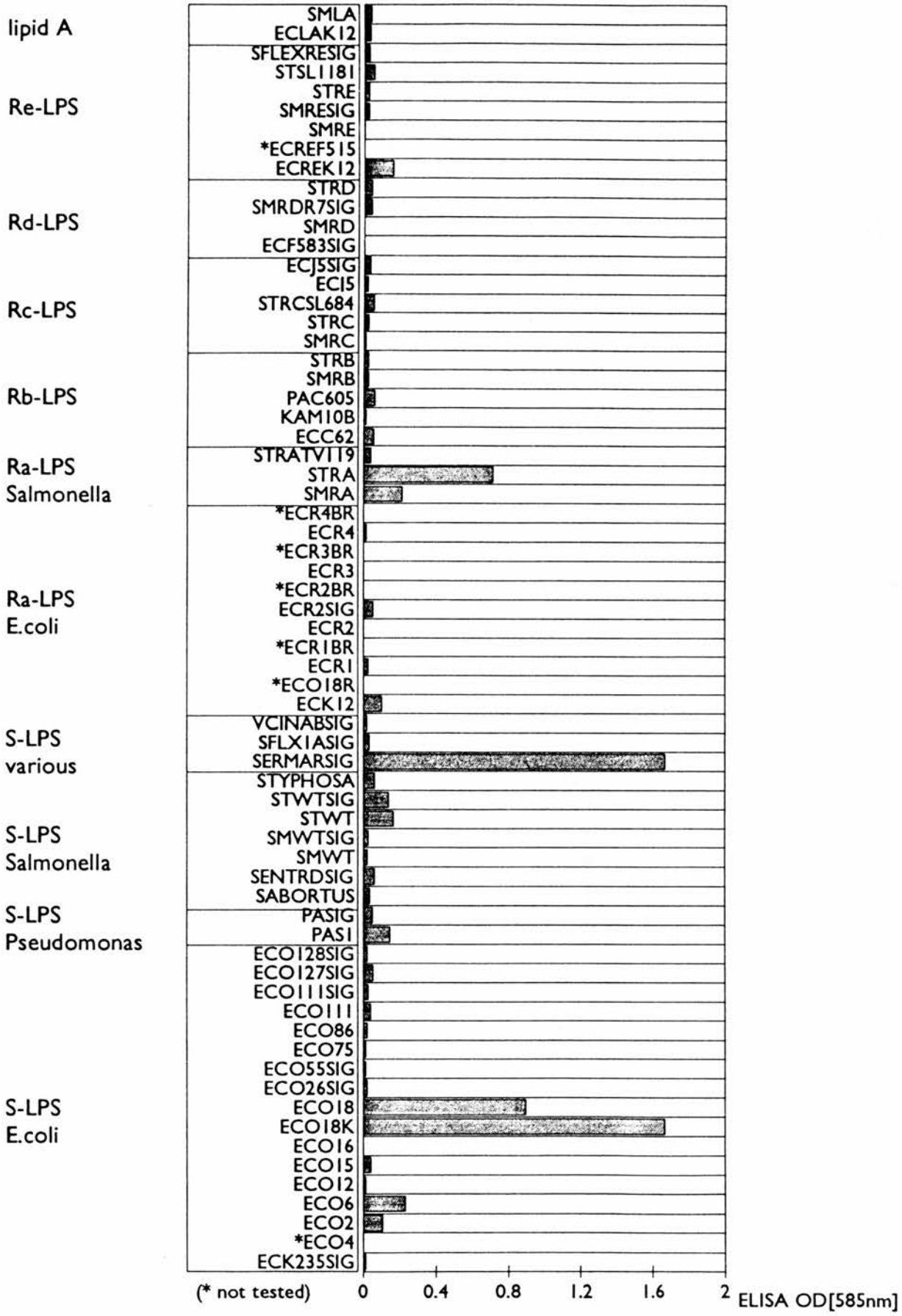


MAb specificity (maximum-binding) screening ELISA

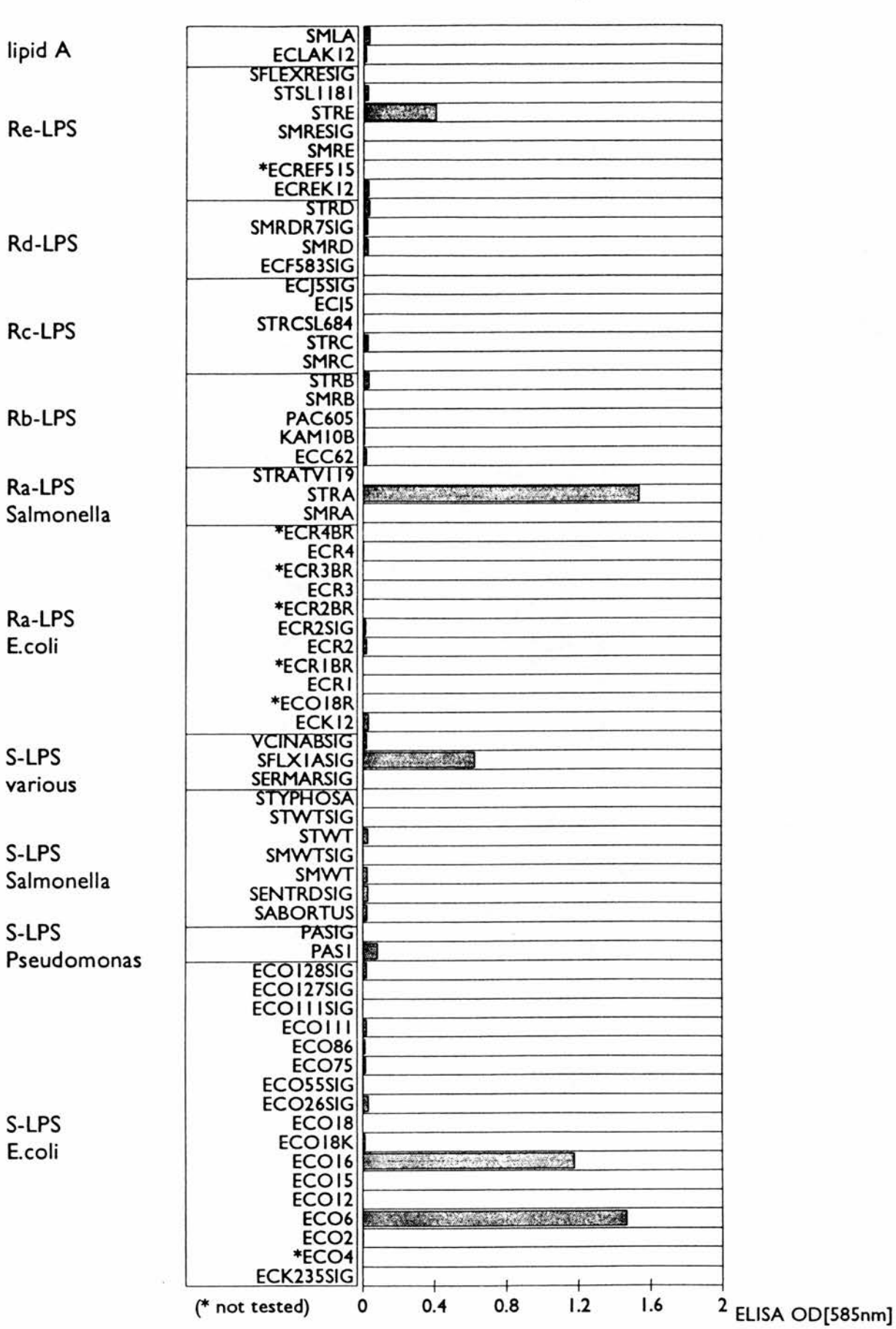


MAb specificity (maximum-binding) screening ELISA

Monoclonal anti-LPS antibody ES-184-2.5.5



MAb specificity (maximum-binding) screening ELISA



MAb specificity (maximum-binding) screening ELISA

Journal of Immunological Methods, 133 (1990) 227–233
Elsevier

JIM 05716

A comparison of immunoblotting, flow cytometry and ELISA to monitor the binding of anti-lipopolysaccharide monoclonal antibodies

D. Nelson, W. Neill and I.R. Poxton

Bacteriology Department, University of Edinburgh Medical School, Teviot Place, Edinburgh EH8 9AG, U.K.

(Received 4 May 1990, revised received 20 June 1990, accepted 25 June 1990)

This study was designed to assess the use of flow cytometry to observe the binding, under physiological conditions, of anti-lipopolysaccharide (LPS) monoclonal antibodies (mAbs) to whole bacteria, and to compare this with the more conventional whole cell ELISA and immunoblotting techniques. The bacteria consisted of two clinical isolates of *E. coli* 018:K1 and 06:K5 and two isogenic mutants of the 018 parent: a non-capsulate (018:K$^-$) and a rough mutant (018rf). Two cross-reactive anti-core mAbs and one 018 0-antigen-specific mAb were used. ELISA and flow cytometry showed that capsule and O-polysaccharide influenced the binding of mAbs to the bacteria, whilst the latter technique demonstrated that sub-populations existed. Immunoblotting showed the two anti-core mAbs to be different, one bound only to core which was not substituted with O-antigen, whilst the other bound both to substituted and unsubstituted core. This comparison for monitoring the binding of anti-LPS mAbs demonstrates the potential use of flow cytometry in bacterial cell surface research, and complements results obtained by ELISA and immunoblotting.

Key words: Flow cytometry; ELISA; Immunoblotting; Lipopolysaccharide; *E. coli*; Monoclonal antibody

Introduction

Lipolysaccharide (LPS) or endotoxin is a major constituent of the outer membrane of all Gram-negative bacteria and is known to be responsible for the range of pathophysiological features of endotoxic shock. In general, LPS consists of three regions: the outer O-polysaccharide – the composition of which varies with the serotype of the organism, the core oligosaccharide – which is less variable, especially in the inner part, and the inner lipid A – a highly conserved structure, responsible for the toxicity and many of the biological activities of LPS (Rietschel et al., 1984).

Limitations of existing therapeutic agents for endotoxic shock have led to active investigation of protective antibodies. It is thought that antibodies directed against the conserved elements of the core oligosaccharide and lipid A region of LPS may be cross-reactive and possess anti-endotoxic properties (Chedid et al., 1968). An area of great debate is whether antibodies can bind to these inner regions of LPS in its natural states either on the bacterial cell surface or bound to serum components in the circulation (Pollack et al., 1989).

Flow cytometry has been used extensively to analyse eukaryotic cell populations (Melamed et

Correspondence to: I.R. Poxton, Bacteriology Department, University of Edinburgh Medical School, Teviot Place, Edinburgh EH8 9AG, U.K.

al., 1979; Muirhead et al., 1985), although its application to bacteria has remained limited. This study was undertaken to assess the use of flow cytometry for monitoring the binding of different anti-LPS monoclonal antibodies (mAbs) to whole bacteria, and to compare this with the more conventional techniques of enzyme-linked immunosorbent assay (ELISA) and immunoblotting.

Materials and methods

Bacteria and culture conditions

Four strains of *Escherichia coli* were kindly supplied by Dr. A.S. Cross, Walter Reed Institute for Army Research, Washington, DC, U.S.A. They consisted of two clinical isolates (018:K1 and 06:K5 serotypes) together with two isogenic mutants from the 018 parents: a non-capsulate mutant (018:K$^-$) and a rough mutant (018rf). Cultures were grown in 100 ml filter-sterilised nutrient broth (Gibco) in 250 ml conical flasks at 37 °C for 16 h in an orbital incubator. Cells were harvested and washed twice in phosphate-buffered saline, pH 7.4 (PBS). Total counts were made in a haemocytometer (Thoma ruling).

Monoclonal antibodies

Monoclonal antibodies (mAbs) were prepared by fusing spleen cells from immune BALB/c mice with NSO myeloma cells by standard techniques (Kipps and Hertzenberg, 1986). The reactivity of the mAbs was determined by an LPS-polymyxin ELISA method as described by Scott and Barclay (1987). The 018-specific mAb was screened by its binding to 018 LPS, while a cocktail of four rough LPS was used as a primary screen for the anti-core mAbs, followed by a broad secondary screen of both rough and smooth LPS. Full details of the immunisation of mice, selection of hybridomas and characterisation of the mAbs are to be published elsewhere. Three mAbs with known specificities in LPS-polymyxin ELISA were selected for this study: mAb 0-1 (specific for 018 O-antigen), and mAbs C-1 and C-2 (both cross-reactive with core epitopes). Supernatant fluids of hybridoma cell cultures grown in RPMI 1640 supplemented with 5% foetal calf serum in 150 cm^2 flasks were used throughout. Cell cultures were grown to maximum cell density and harvested at 50% cell viability. These three hybridomas gave yields of approximately 50 μg/ml.

Preparation of LPS

LPS was prepared from whole washed bacteria by the Proteinase K method of Hitchcock and Brown (1983). The method is described in detail by Hancock and Poxton (1988). Briefly, bacteria grown overnight were washed twice in PBS and adjusted to an A_{525} between 0.5 and 0.6. After centrifugation, the pellet from 1.5 ml of the bacterial suspension was suspended in 50 μl of sample buffer (2% SDS, 4% 2-mercaptoethanol, 10% glycerol, 0.002% bromophenol blue in 1 M Tris-HCl pH 6.8) and heated for 10 min at 100 °C. Proteinase K (25 μg in 10 μl sample buffer: protease Type X1, Sigma) was added, and the mixture incubated at 60 °C for 60 min.

SDS-PAGE

SDS-PAGE was performed on 14% acrylamide slab gels with the Laemmli buffer system (Laemmli, 1970). Samples (10 μl for silver stain, or 20 μl for immunoblotting) of the proteinase K LPS extracts were loaded on to the gels. The LPS separating gels were stained with silver by a method developed by Tsai and Frasch (1982), modified by Hancock and Poxton (1988). Briefly, gels were fixed overnight in 200 ml of 25% propan-2-ol, 7% acetic acid followed by oxidation in freshly prepared periodic acid. After frequent washing over 4 h in distilled water, fresh ammoniacal silver nitrate solution (1.4 ml of ammonia solution, 21 ml of 0.36% NaOH, 4 ml of 19.4% $AgNO_3$ and distilled water to 100 ml) was added for 15 min. Following at least four washes in distilled water over 40 min, gels were transferred to fresh 0.005% citric acid in 200 ml of 0.019% formaldehyde at 25 °C. On development gels were washed repeatedly in distilled water.

Immunoblot analysis

This was based on the method of Towbin et al. (1979) as described by Hancock and Poxton (1988) with Bio-Rad buffers and substrate. Electrophoretic transfer of LPS to nitrocellulose sheets of a 0.2 μm pore size (Schleicher and Schuell) was

performed at 12 V in electroblotting apparatus for 16 h at 4°C. After transfer, the nitrocellulose membrane was washed in Tris-buffered saline (TBS) for 10 min and blocked with 3% gelatin to prevent non-specific binding of antibodies. A 1 in 10 dilution of culture supernatant fluids (shown previously to give optimum staining) was added for 3 h, followed by two 10 min washes in Tween Tris-buffered saline (TTBS). After a 60 min treatment with an anti-mouse IgG/M/A -horseradish peroxidase conjugate (Zymed), diluted 1 in 500, membranes were washed in TTBS as before. Freshly prepared HRP colour development reagent was added and left for 30 min before washing in distilled water.

Flow cytometry

Washed, overnight cultures of *E. coli* were resuspended to an A_{525} of between 0.5 and 0.6, equivalent to approximately 1×10^8 cfu/ml. Suspensions (1 ml) were centrifuged at $10{,}000 \times g$ in a Microfuge (Beckman) for 2 min and the pellets resuspended in 1 ml of mAb culture supernate, diluted 1 in 10 in dilution buffer (see ELISA methodology), and incubated for 60 min at 37°C. Samples were washed twice in PBS followed by addition of 0.5 ml, sheep FITC-conjugated anti-mouse IgG (ICN) diluted 1 in 100 in dilution buffer. After a further incubation of 60 min at 37°C, samples were washed twice in PBS and resuspended in 1 ml PBS containing 0.5% formaldehyde. Prepared samples, diluted 1 in 50 in PBS, were analysed in an EPICS 'C' (Coulter Electronics) flow cytometer equipped with a 5 W argon ion laser operating at 500 MW output and exciting at 488 nm. Cells were passed through the beam at approximately 500/s from a standard 76 μm flow cell tip. With the use of gates on the log forward angle light scatter (LFLS) signal, both background noise and clumps of cells were excluded from the analysis. Cells stained with FITC conjugate but no primary antibody provided a background staining level which was set at 1% ± 0.5% by adjusting the voltage applied to the green fluorescence log (GFL) photomultiplier tube. A total of 50,000 cells were analysed from each sample and the percentage of cells exhibiting positive staining on GFL was calculated by the EPICS 'Stat Pack' programme.

ELISA

Whole cell ELISA was based on the method of Scott (1988). ELISA strips (Immuno module Polysorp F8, Nunc) were coated with washed bacteria (100 μl/well) from a 16 h culture diluted with coating buffer (0.05 M carbonate/bicarbonate pH 9.6 containing 0.02% sodium azide) to a concentration of 2×10^7 cells/ml. Coating was promoted by centrifugation at $630 \times g$ for 4 min and leaving overnight at room temperature. Plates were washed four times with PBS pH 7.2, containing Tween 20 (0.05% v/v) and sodium azide (0.02% w/v) and were then post-coated with bovine serum albumin (5% w/v BSA) and sodium azide (0.02% w/v) in PBS at 100 μl/well followed by overnight incubation at room temperature. After washing

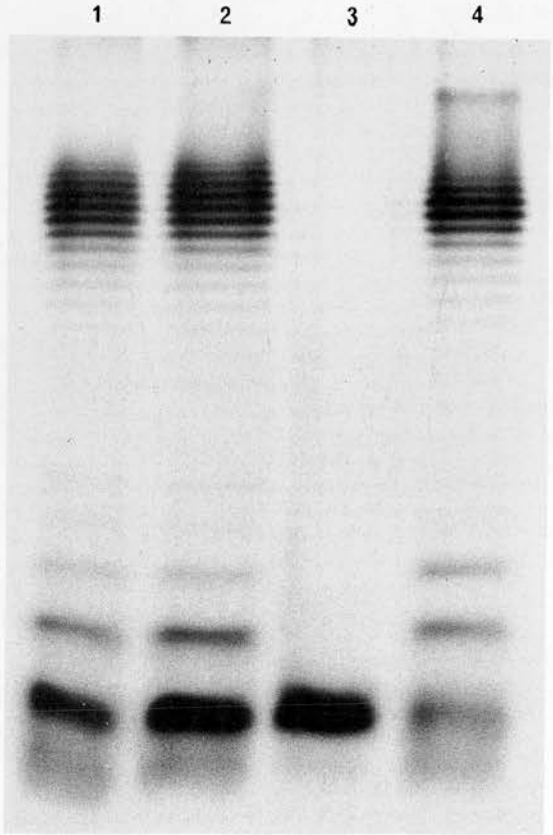


Fig. 1. Silver-stained PAGE of proteinase K digests of *E. coli* grown in filter-sterilised nutrient broth at 37°C for 16 h in an orbital incubator. Track *1*, 018:K1; *2*, 018:K$^-$; *3*, 018rf; and *4*, 06:K5.

four times, plates were rinsed in distilled water and stored at −20 °C until used.

Dilutions of mAb supernatant fluids and conjugate were made in dilution buffer consisting of PBS, with Tween 20 (0.05% v/v), BSA (0.5% w/v), polyethylene-glycol 6000 (4% w/v) and sodium azide (0.02%). Antibody dilutions were added to coated plates at 100 µl/well in triplicate and plates incubated at 37 °C for 90 min before washing four times. Urease-conjugated sheep anti-mouse Ig (Seralab) was diluted 1 in 500, added at 100 µl/well and plates incubated for a further 90 min at 37 °C. Plates were washed four times and rinsed with distilled water before addition of urease substrate (Seralab) at 100 µl/well. Plates were incubated at room temperature and reactions stopped by addition of thimerosal (1%) in distilled water (20 µl/well). Absorbances (A) were read at 590 nm in a Titertek Multiscan.

Results

Silver stain

The silver-stained SDS-PAGE profiles of LPS from the four *E. coli* strains are shown in Fig. 1. The LPS from the three smooth strains showed the characteristic ladder pattern, with each band differing in M_r by one repeating unit of O-antigen. The rough LPS lacked any of the high molecular mass bands which corresponded to LPS substituted with O-polysaccharide chains.

Immunoblots on LPS

Fig. 2. shows the reaction of the anti-LPS antibodies with the four LPS preparations in immunoblots. The 018, O-antigen-specific antibody (mAb 0-1), bound strongly to the high M_r bands of both the smooth 018 strains but not to the 06 LPS (Fig. 2*a*). It also revealed a small amount of high molecular mass O-antigen leaking from the rough mutant of 018. Of the two anti-core mAbs, mAb C-1 bound only to the fast migrating species that corresponded to the core glycolipid demonstrated on silver-stained gels (Fig. 2*b*). Staining was strongest for the rough *E. coli* LPS and weakest for the *E. coli* 06. The reactivity of mAb C-2 again was to the fast migrating core region, but there was also binding to the high M_r, O-antigen bearing molecular species of the smooth strains (Fig. 2*c*).

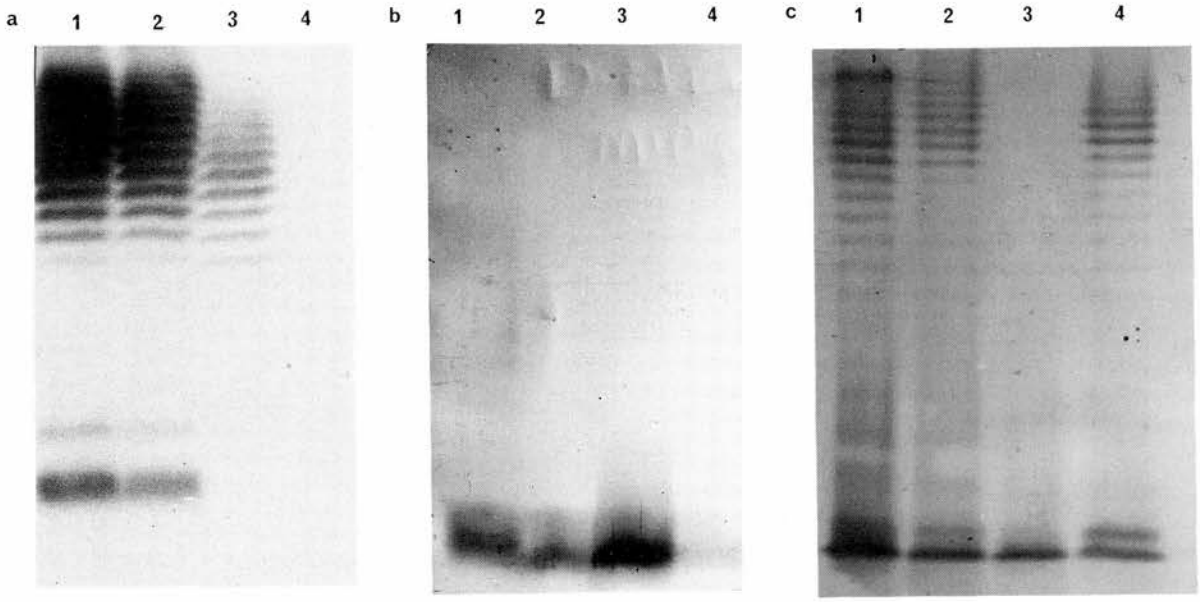


Fig. 2. Immunoblots of proteinase K extracts of *E. coli* strains arranged as in Fig. 1, probed with an anti-018-specific mAb, 0-1 (*a*), and two cross-reactive, core-specific mAbs, C-1 (*b*) and C-2 (*c*).

Flow cytometry on whole bacteria

Fig. 3. shows the binding of anti-LPS mAbs to whole cells by flow cytometry. The reactivity of the 018-specific mAb, mAb 0-1 is shown in Fig. 3*a*. The percentage of bacteria exhibiting positive fluorescence above background levels was in excess of 70% for the two smooth 018 strains, whilst the *E. coli* 06 cells showed negligible binding. The non-capsulate 018 strain showed two distinct populations of cells, indicative of differences in amount of antibody bound. The presence of small amounts of O-antigen on the rough mutant is again evident. The three smooth strains bound anti-core mAb C-1 and mAb C-2 antibodies at less than 10% above background levels (Figs. 3b and 3c). However, binding of mAb C-1 to 018:K$^-$ was almost double that of 018:K1. Percentage binding to the rough mutant by mAb C-1 was 84% compared to a much lower figure of 14% for mAb C-2.

ELISA on whole bacteria

The binding activities of the three mAbs to whole cells in a urease ELISA system are shown in Fig. 4. Reactivity of the 018-specific mAb 0-1 mirrored results of immunoblotting and flow cy-

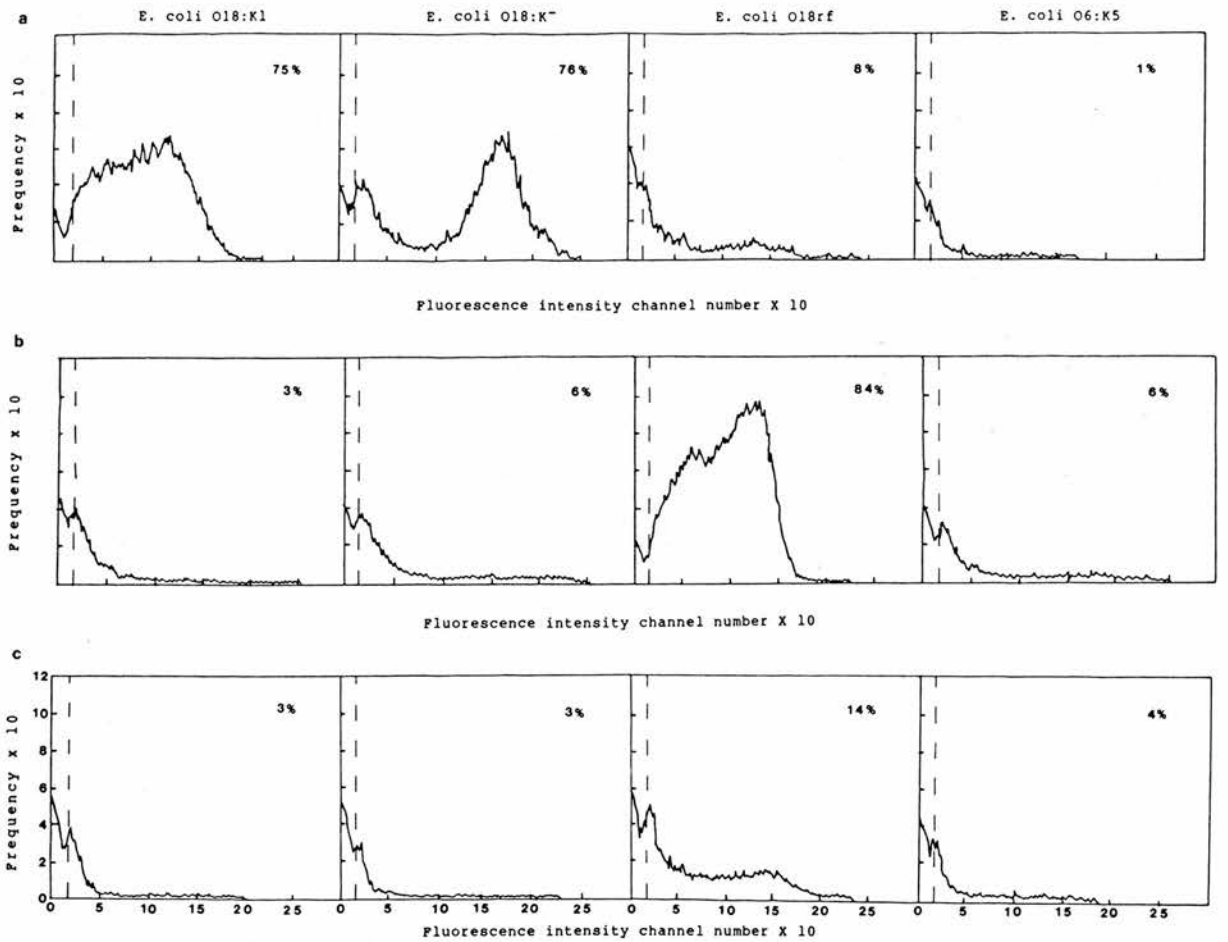


Fig. 3. Flow cytometry. Green fluorescence intensity histograms of culture supernates of three mAbs against whole cells of four *E. coli* strains, (018:K1; 018:K$^-$; 018rf and 06:K5). *a*, *b* and *c* represent binding of the anti-018-specific mAb 0-1, and two cross-reactive, core-specific mAbs, C-1 and C-2 respectively. Percentage values represent bacteria exhibiting positive fluorescence above background levels (indicated by vertical dotted line).

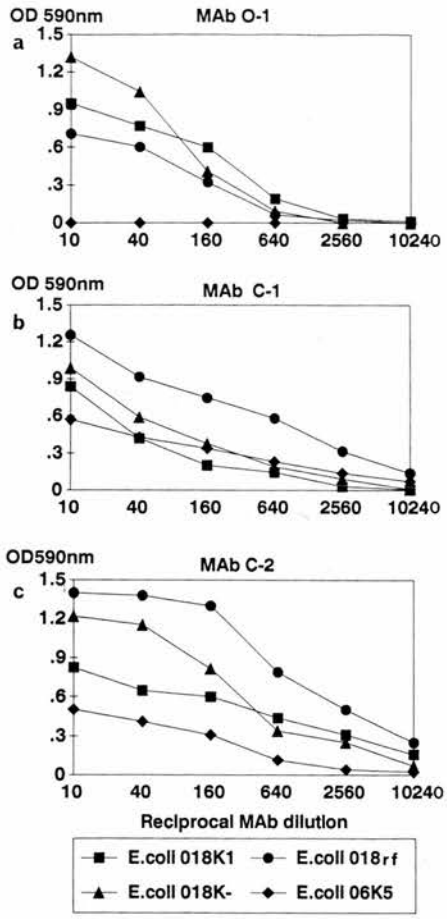


Fig. 4. Binding activities of culture supernates of three mAbs against whole cells of four *E. coli* strains in ELISA: 018:K1; 018: K^-; 018rf and 06:K5. *a*, *b* and *c* represent binding of the anti-018-specific 0-1, and two cross-reactive, core-specific mAbs C-1 and C-2, respectively.

tometry. Smooth 018 strains had the highest A_{590} readings, whilst binding to the rough mutant reflected the presence of some leaking O-antigen. The binding of the mAb at high concentrations was much stronger to the non-capsulate strain than to the capsulate parent. The three smooth *E. coli* strains all showed lower A_{590} values compared to the rough *E. coli* when probed with dilutions of the two anti-core mAbs, C-1 and C-2. Again, the 018 non-capsulate strain showed stronger affinity for the anti-core mAbs compared to its capsulate parent.

Discussion

The individual techniques used in this study provide a means of assessing the binding specificity of anti-LPS mAbs. In combination, a much more comprehensive picture can be built up of the accessibility of endotoxin sites to antibody. Immunoblot patterns of proteinase K LPS extracts against mAbs provide general information regarding their specificity. Indeed, the different reactivity patterns of the two anti-core antibodies was only apparent by immunoblotting. The ladder pattern or single core band effect reflected the binding of C-2 to substituted and unsubstituted core, and C-1 to unsubstituted core material only. It should be noted that the binding of mAbs to blotted LPS does not suffer from the loss or denaturation of epitopes in the same way as proteins.

Results obtained by flow cytometry and ELISA on whole bacteria showed that the absence of O-polysaccharide side chains on the rough mutant appeared to increase the accessibility of core LPS to antibodies, resulting in better binding than to smooth strains. This shielding effect by O-antigenic chains of LPS was also demonstrated by Gigliotti and Shenep (1985). A comparison of the ability of 018:K1 and 018:K^- to bind antibody suggests that capsule may have a role in masking LPS binding sites. Flow cytometry, however, also reveals further information about antibody binding to LPS not detected in ELISA. Within a population of cells showing positive fluorescence an indication of the affinity of an antibody for its antigen can be obtained. The presence of two distinct populations of *E. coli* 018:K^- cells when probed with 0-1 indicates possible differences in structure or amount of LPS.

Flow cytometry offers a means of investigating surface properties of individual cells in large numbers with great speed and efficiency. The technique also has the added advantage of analysing cells in their natural form, devoid of potential distorting influences present in other methods. However, despite the unequivocal potential of flow cytometric analysis of bacteria, it remains an under-used tool, still in its infancy. Phillips and Martin (1988) used flow cytometry for the specific detection of bacteria in aqueous samples. Their

work established problems with background noise as a result of stray light scatter in the optical system, or possibly signals from sub-micron particles in sheath fluids and PBS which had not been removed by filtration. Selective gating near the origin of the cytogram was not feasible in Philips and Martin's study, although it was used in our study to eliminate background noise.

ELISA is an established method for the detection of antibodies to bacteria, offering advantages of speed, flexibility and quantitative accuracy. The apparent higher binding of anti-LPS mAbs to smooth cells in ELISA than to flow cytometry may reflect the better sensitivity of the whole cell ELISA. However, in contrast to flow cytometry, recent data (not shown) suggested that in some instances the expression of coated whole cells on ELISA plates differs from whole cells in suspension, probably due to their alteration during binding manipulations. Our results suggest that ELISA has tended to over-emphasise the binding of anti-core mAbs to whole cells. Indeed, Aydintug et al. (1989) established that both the physical state of bacteria and type of assay used affect the cross-reactivity of an antibody.

Acknowledgements

We are extremely grateful to all collaborators: Drs. G.R. Barclay, B.B. Scott, C.V. Prowse and colleagues of S.E. Scotland Blood Transfusion Service for all the help in the development of screening assays and selection of mAbs, to Loraine McMillan and Dr. Keith James and colleagues in the Department of Surgery where the fusions were performed, to Frances McLoughlin of this department for help with the development of the flow cytometry and to Drs. M. Shreier and F. Di Padova of Sandoz, Basel, Switzerland for their help and encouragement.

Sandoz is gratefully acknowledged for financing this work.

References

Aydintug, M., Inzana, T., Letonja, T., Davis, W. and Corbeil, L. (1989) Cross-reactivity of monoclonal antibodies to *Escherichia coli* J5 with heterologous gram-negative bacteria and extracted lipopolysaccharides. J. Infect. Dis. 160, 846.

Chedid, L., Parant, M., Parant, F. and Boyer, F. (1968) A proposed mechanism for natural immunity to enterobacterial pathogens. J. Immunol. 100, 292.

Gigliotti, F. and Shenep, J.L. (1985) Failure of monoclonal antibodies to core glycolipid to bind intact smooth strains of *Escherichia coli*. J. Infect. Dis. 151, 1005.

Hancock, I.C. and Poxton, I.R. (1988) Bacterial Cell Surface Techniques. Wiley, Chichester, U.K.

Hitchcock, P.J. and Brown, T.M. (1983) Morphological heterogeneity among Salmonella lipopolysaccharide chemotypes in silver-stained polyacrylamide gels. J. Bacteriol. 154, 269.

Kipps, T.J. and Hertzenberg, L.A. (1986) Schemata for production of monoclonal antibody-producing hybridomas. In: D.M. Weir (Ed.), Handbook of Experimental Immunology, 4th edn, Vol. 4, Chap. 108. Blackwell, Oxford.

Laemmli, U.K. (1970) Cleavage of structural proteins during assembly of the head protein of bacteriophage T4. Nature 277, 680.

Melamed, M.R., Mulaney, P.F. and Mendelsohn, M.M. (1979) Flow cytometry and sorting. Wiley, New York.

Muirhead, K.A., Horan, P.K. and Posk, G. (1985) Flow cytometry: Present and future. Biotechnology 3, 337.

Phillips, A.P. and Martin, K.L. (1988) Limitations of flow cytometry for the specific detection of bacteria in mixed populations. J. Immunol. Methods 106, 109.

Pollack, M., Chia, J.K.S., Koles, N.L., Miller, M. and Guelde, G. (1989) Specificity and cross-reactivity of monoclonal antibodies reactive with the core and lipid A regions of bacterial lipopolysaccharide. J. Infect. Dis. 159, 168.

Rietschel, E.T., Wollenweber, H.-W., Brade, H., Zahringer, V., Lindner, B., Seydel, U., Bradaczek, H., Barnnickel, G., Labischinski, H. and Giesbrecht, P. (1984) Structure and conformation of the lipid A component of lipopolysaccharides. In: E.T. Rietschel (Ed.) Handbook of Endotoxin, Vol. 1, Chemistry of Endotoxin. Elsevier, Amsterdam, p. 187.

Scott, B.B. (1988) Identification and functional evaluation of cross-reactive antibodies to gram-negative bacterial endotoxin in a blood donor population. PhD thesis, Council for National Academic Awards, London.

Scott, B.B. and Barclay, G.R. (1987) Endotoxin-polymyxin complexes in an improved enzyme-linked immunosorbent assay for IgG antibodies in blood donor sera to gram-negative endotoxin core glycolipids. Vox Sang. 52, 272.

Towbin, H., Staehelin, T. and Gordon, J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. U.S.A. 76, 4350.

Tsai, C.-M. and Frasch, C.E. (1982) A sensitive silver stain for detecting lipopolysaccharide in polyacrylamide gels. Anal. Biochem. 119, 115.

HNJ CHARS: 000038422
MSG: 75300 — Corrected
16-OCT-91 17:17:521

Paper 102

Journal of General Microbiology (1991), **137**, 0000–0000. *Printed in Great Britain*

Monoclonal antibodies as probes for detecting lipopolysaccharide expression on *Escherichia coli* from different growth conditions

DAVID NELSON, ANDREW J. BATHGATE and IAN R. POXTON*

Department of Medical Microbiology, University of Edinburgh Medical School, Teviot Place, Edinburgh, EH8 9AG, UK

(*Received 10 June 1991; revised 22 August 1991; accepted 16 September 1991*)

Monoclonal antibody (mAb) probes were used to investigate the expression of lipopolysaccharide (LPS) on four *Escherichia coli* strains, grown under a variety of conditions in batch culture which mimicked some of the *in vivo* environmental conditions of an infected host. Techniques of silver staining, immunoblotting, whole cell ELISA and flow cytometry were all used to monitor the expression of LPS on the bacteria and the binding of the anti-LPS mAbs. Growth in heat-inactivated sheep serum and magnesium-depleted conditions demonstrated increased expression of LPS core and subsequent increased binding of anti-core mAbs. Magnesium-depleted conditions also resulted in decreased production of O-polysaccharide material. Iron-depleted bacteria showed only minor changes in LPS expression, although increased binding of anti-core mAbs was observed. Nitrogen-deficient/high-carbon conditions, chosen to promote capsule production, resulted in increased expression of O-polysaccharide and decreased binding of anti-core mAbs.

Introduction

The potential of antibody therapy for the prevention and treatment of Gram-negative sepsis and endotoxaemia with anti-lipopolysaccharide (LPS) antibodies has been intensively investigated recently (Dunn *et al.*, 1986). Much attention has been focussed on the production and characterization of monoclonal antibodies (mAbs) to common epitopes of endotoxin in the LPS core. There are many reports in the literature of the development of such mAbs (Ziegler *et al.*, 1991; de Jongh-Leuvenink, 1990; Mayoral & Dunn, 1990; Pollack *et al.*, 1989). The therapeutic potential of anti-LPS mAbs is, however, open to much debate (Heumann *et al.*, 1991; Aydintug *et al.*, 1989; Chia *et al.*, 1989). Concern exists as to whether it is possible for these antibodies to bind to sites deep within the LPS molecule, either when the LPS is bound to the bacterium or to host components such as high density lipoprotein and endothelial surfaces, or 'free' in micelles. A further complication exists by which the heterogeneity of O-polysaccharide chain length and degree of substitution of the LPS core may affect antibody accessibility (Gigliotti & Shenep, 1985).

We have shown recently that it is possible, with a combination of ELISA and flow cytometry, to use monoclonal antibodies to detect LPS expression on whole bacteria (Nelson *et al.*, 1990). Results showed, however, that although anti-O-polysaccharide mAbs could readily be observed binding to bacteria by both techniques, anti-core mAbs could be shown to bind significantly only by the sensitive ELISA and not by flow cytometry.

It is now well recognized that the growth environment of bacteria greatly influences the phenotypic expression of surface characters (Smith *et al.*, 1991; McGroarty & Rivera, 1990; Kelly *et al.*, 1989; Morse *et al.*, 1983). This study attempts to investigate with mAb probes the expression of LPS on bacteria cultured in conditions which mimic those *in vivo*.

Methods

Bacteria. Four strains of *Escherichia coli* were kindly supplied by Dr A. S. Cross, Walter Reed Institute for Army Research, Washington, DC, USA. They consisted of two clinical isolates (O18:K1 and O6:K5 serotypes) together with two isogenic mutants from the O18 parent: a non-capsulate mutant (O18:K$^-$) and a rough mutant (O18Krf).

Culture conditions. The following growth media were used. (*a*) Nutrient broth (Gibco), filter-sterilized. (*b*) Magnesium-depleted medium. This was prepared as a modification of the Malka minimal medium of Robert-Gero *et al.* (1970) as follows: solution A, Na_2HPO_4 (73·4 mg ml^{-1}), KH_2PO_4 (32·4 mg ml^{-1}); solution B, $MgSO_4.7H_2O$ (20·5 mg ml^{-1}); solution C, 20% (w/v) glucose; solution D, $FeSO_4.7H_2O$ (1·83 mg ml^{-1}) in sterile distilled water to which one drop of concentrated hydrochloric acid was added; solution E $(NH_4)_2SO_4$ (50·0 mg ml^{-1}). All chemicals were from BDH. Solutions were prepared with sterile distilled water and were filter sterilized. All

solutions except C were stored over chloroform. To prepare 1 litre of Malka, 20 ml A, 20 ml B, 20 ml C, 1 ml D and 20 ml E were added to 919 ml of sterile distilled water. Magnesium-depleted medium containing 0·17 mmol l^{-1} was prepared by the addition of only a 1% vol. (0·2 ml) of solution B as used for the standard minimal medium. (*c*) Nitrogen-deficient high-carbon medium. This was prepared following the method of Sutherland & Wilkinson (1965) and contained: 1 g yeast extract (Oxoid); 1 g Casamino acids (Difco technical grade); 10 g Na_2HPO_4; 3 g KH_2PO_4; 0·2 g $MgSO_4.7H_2O$; 11 g K_2SO_4; 1 g NaCl; 0·01 g $CaCl_2.2H_2O$; and 0·01 g $FeSO_4.7H_2O$. The volume was made up to 1000 ml with sterile distilled water and autoclaved. To this, 20% (w/v) filter-sterilized glucose was added to give a final concentration of 2% (w/v) glucose. (*d*) Iron-depleted medium. This was prepared by the addition of 150 μmol 2,2'-dipyridyl l^{-1} to Gibco nutrient broth. (*e*) Heat-inactivated sheep serum (obtained from the Moredun Research Institute, Edinburgh, UK): the serum was filter-sterilized (0·45 μm pore size) and heat-inactivated at 56 °C for 60 min and stored at −20 °C.

Exponential phase starter cultures were prepared by growth in nutrient broth at 37 °C for 4 h before harvesting and washing twice in phosphate-buffered saline (PBS; 0·15 M-NaCl, 50 mM-potassium phosphate buffer), pH 7·4. A 1% inoculum was added to each growth medium. Cultures were grown at 37 °C in 100 ml of each medium to early stationary phase in an orbital incubator prior to harvesting and washing as above.

Monoclonal antibodies. mAbs were prepared by fusing spleen cells from immune BALB/c mice with NS-O myeloma cells by standard techniques (Kipps & Hertzenberg, 1986). The reactivity of the mAbs was determined by the LPS–polymyxin ELISA method of Scott & Barclay (1987) as described by Nelson *et al.* (1990). Full details of the immunization of mice, selection of hybridomas and characterization of mAbs are to be published elsewhere. Three mAbs with known specificities in LPS–polymyxin ELISA were selected for this study: mAb O-1, specific for O18 O-antigen, and two cross-reactive anti-core mAbs (C-1 and C-2), reactive to either core not substituted with O-antigen (C-1) or to both substituted and unsubstituted core material (C-2) (for more details see Nelson *et al.*, 1990). Supernatant fluids of hybridoma cell cultures grown in RPMI 1640 supplemented with 5% (v/v) foetal calf serum in 150 cm^2 flasks were used throughout. Cell cultures were grown to maximum cell density and harvested at 50% cell viability. These three hybridomas gave yields of approximately 50 μg ml^{-1}.

Preparation of LPS. LPS was prepared from whole washed bacteria by the proteinase K method of Hitchcock & Brown (1983) as described by Hancock & Poxton (1988). For each growth condition the density of washed bacteria was adjusted to an OD_{525} of 0·5 prior to proteinase K treatment. This allowed direct comparison of cells from each growth medium.

PAGE. PAGE was performed on 14% (w/v) acrylamide slab gels with the Laemmli buffer system (Laemmli, 1970), except SDS was omitted from the stacking and separating buffers. Samples (10 μl for silver stain, or 20 μl for immunoblotting) of the proteinase K LPS extracts were loaded onto the gels. The LPS separating gels were stained with silver by a method developed by Tsai & Frasch (1982), as modified by Hancock & Poxton (1988).

Immunoblot transfer. LPS separated by PAGE was electroblotted to nitrocellulose by the method of Towbin *et al.* (1979) as described by Hancock & Poxton (1988) with Bio-Rad buffers and substrate. Nitrocellulose membrane of 0·2 μm pore size was obtained from Schleicher and Schuell and an anti-mouse IgG–horseradish-peroxidase conjugate used (ICN). Antibody culture supernates were diluted 1 in 10.

Diluents and buffers used in ELISA. (1) Coating buffer consisted of 0·05 M-carbonate/bicarbonate, pH 9·6. (2) Post-coat buffer consisted of PBS (pH 7·2) containing 2% (w/v) bovine serum albumin (BSA) (ICN). (3) Wash buffer consisted of PBS containing 0·05% (v/v) Tween 20. (4) Dilution buffer consisted of PBS containing 0·05% (v/v) Tween 20, 0·5% BSA and 4% (w/v) polyethylene glycol 6000 (Sigma).

All solutions contained 0·02% sodium azide.

ELISA procedure. ELISA strips (Immuno module Polysorp F8, Nunc) were coated with washed bacteria (100 μl per well) after measuring the OD_{540} and diluting with coating buffer to a concentration of 2×10^7 cells ml^{-1}. Coating was promoted by centrifugation at 630 *g* for 4 min and leaving overnight at room temperature. Plates were washed four times in wash buffer, before post-coating with post-coat buffer at 100 μl per well overnight at room temperature. After further washing, plates were rinsed in distilled water and stored at −20 °C until used. mAb supernatant fluids were diluted 1 in 10, 40, 160 and 640 in dilution buffer and added to coated microplates at 100 μl per well in triplicate. Plates were incubated at 37 °C for 90 min before washing four times with wash buffer. Urease-conjugated sheep anti-mouse Ig (SeraLab) was diluted 1 in 500, added at 100 μl per well and plates were incubated for a further 90 min at 37 °C. Plates were washed four times and rinsed in distilled water before urease substrate (SeraLab) at 100 μl per well was added. Plates were incubated for 60 min at room temperature and reactions stopped by adding 1% (w/v) thimerosal (Sigma) in distilled water (20 μl per well). The *A* of wells was read on an automated microplate reader Titertek Multiscan (MC, Flow Laboratories). Final results were expressed after subtraction of the absorbance readings of negative control wells (coated with BSA post-coat only) for each mAb.

Flow cytometry. Flow cytometry was based on the method described by Nelson *et al.* (1990). Briefly, washed cultures of bacteria were resuspended to a concentration of about 1×10^8 cells ml^{-1}. Pellets from 1 ml were resuspended in 1 ml of mAb culture supernate, diluted 1 in 10 in dilution buffer (see ELISA methodology), and incubated for 60 min at 37 °C. After washing twice in PBS, 0·5 ml sheep FITC-conjugated anti-mouse IgG (ICN), diluted 1 in 100 in dilution buffer, was added and incubated for 60 min at 37 °C. After further washing in PBS, the pellet was resuspended in PBS containing 0·5% formaldehyde. Samples were diluted 1 in 50 in PBS and analysed in an EPIC 'C' (Coulter Electronics) flow cytometer with a 5 W argon ion laser operating at 500 mW and exciting at 488 nm. A total of 50000 cells at 500 cells s^{-1} were passed through the beam from a 76 μm tip. Background noise and clumps of cells were excluded by a gate on the log forward angle light scatter. The percentage of cells exhibiting positive staining was calculated with the EPICS 'Stat Pack' program.

Results

Silver-staining and immunoblot analysis of LPS

Figs 1–3 represent silver-stained PAGE profiles of LPS and their corresponding immunoblots from four *E. coli* strains grown under different batch culture growth conditions. The three smooth strains showed the characteristic ladder pattern, each step up representing LPS substituted with a progressively increasing number of O-polysaccharide repeating oligosaccharide units. These were missing from the rough mutant. The effect of each growth condition on the expression of LPS was compared to nutrient broth. Silver-stained profiles of LPS of

Table 1. *ELISA results of the three anti-LPS mAbs titrated against whole cells of E. coli O18:K1 and O18:K⁻ grown in five different media*

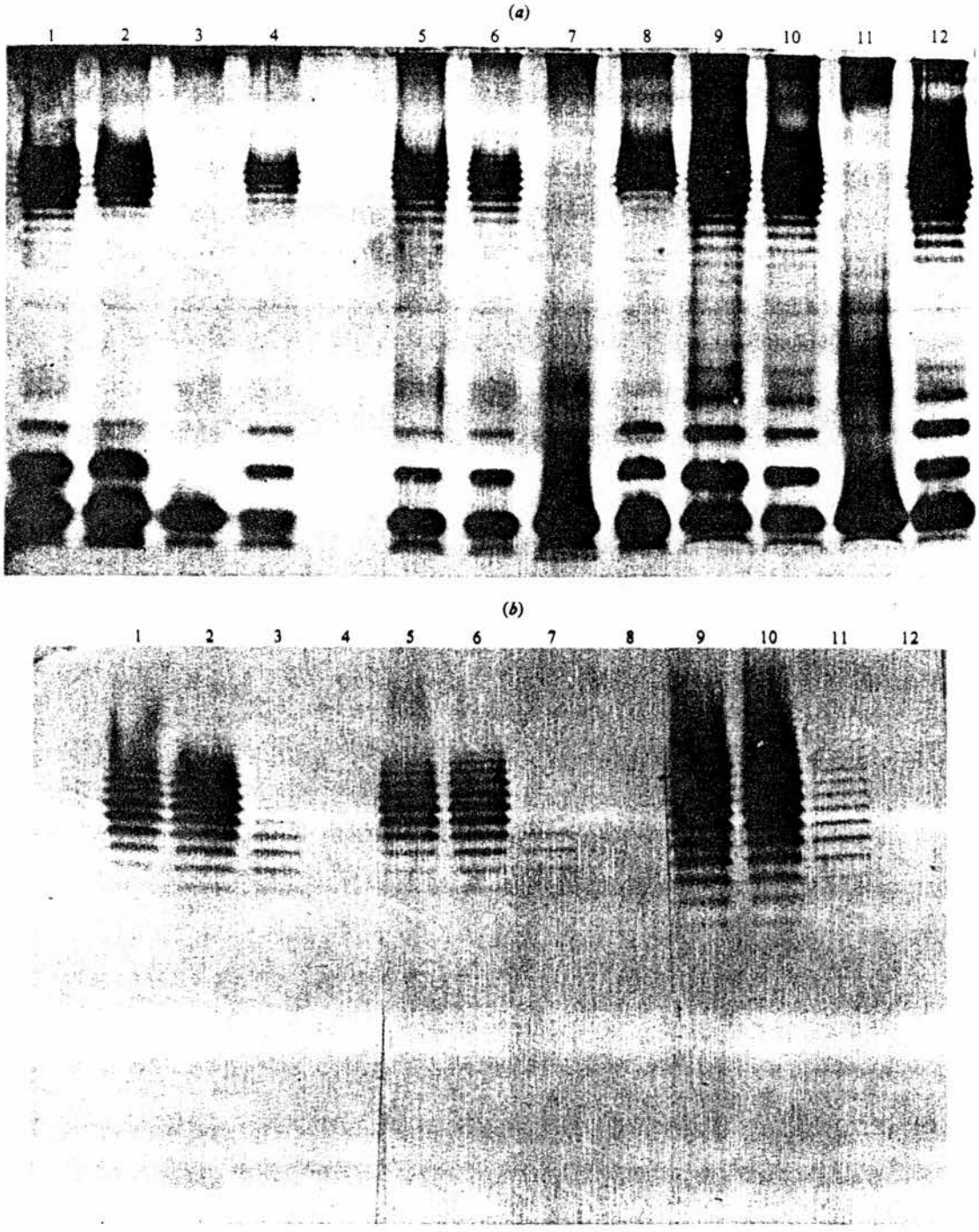
mAb	Growth medium / *E. coli* ...	ELISA results: A_{max}* O18:K1	A_{max}* O18:K⁻	D_{50}† O18:K1	D_{50}† O18:K⁻
O-1	Nutrient broth	1·26	1·34	120	260
	Nitrogen-deficient/ high-carbon	1·31	1·33	145	280
	Iron-depleted	1·19	1·24	105	110
	Serum	1·31	1·41	150	210
	Magnesium-depleted	1·39	1·19	120	75
C-1	Nutrient broth	0·32	0·55	37	36
	Nitrogen-deficient/ high-carbon	0·17	0·20	23	40
	Iron-depleted	0·65	0·82	32	90
	Serum	0·97	1·15	140	180
	Magnesium-depleted	0·88	1·18	66	120
C-2	Nutrient broth	0·45	0·51	40	40
	Nitrogen-deficient/ high-carbon	0·08	0·23	50	60
	Iron-depleted	0·62	0·98	56	36
	Serum	0·73	1·24	270	370
	Magnesium-depleted	0·87	1·01	34	200

* Maximum A_{590} for the 1 in 10 dilution (first dilution) of antibody.
† The dilution factor when the absorbance is 50% of the maximum.

proteinase-K-digested whole cells grown in nutrient broth, iron-depleted and nitrogen-deficient/glucose-enriched media are shown in Fig. 1(*a*). LPS from iron-depleted cells showed only minor changes compared to the LPS from cells grown in nutrient broth, whilst cells grown in a nitrogen-deficient/glucose-enriched medium showed an increased expression of both mid-range and high molecular mass bands. Immunoblotting of trans-blotted gels against mAb O-1 (Fig. 1*b*), demonstrates the specificity for the O-antigen of *E. coli* O18 and small amounts of high molecular mass O-antigen leaking from the rough mutant of O18. The immunoblot again illustrates greater expression of O-antigen as well as an overall increase in O-polysaccharide chain length for cells grown under nitrogen deficiency. Probing with C-2, an anti-core mAb reactive against both substituted and unsubstituted core material showed no significant differences between growth conditions (Fig. 1*c*). PAGE of LPS from the four *E. coli* strains grown in magnesium-depleted medium showed a significant increase in the expression of unsubstituted core-glycolipid material and other low molecular mass bands (Fig. 2*a*). Immunoblotting with mAb O-1 showed similar expression of O-antigen for both nutrient broth and magnesium-depleted conditions (Fig. 2*b*), whilst probing with mAb C-2 further highlighted a pronounced increase in core LPS (Fig. 2*c*). Growth in sheep serum resulted in greater expression of both the fast migrating core region, as well as high molecular mass O-antigen-bearing molecular species of the smooth strains compared to growth in nutrient broth (Fig. 3*a*). Immunoblot analysis with mAb C-1, reactive only with unsubstituted core material, and C-2, demonstrated better expression of predominantly rough form LPS when grown in serum (Fig. 3*b*, *c*).

ELISA on whole bacteria

The binding activities of the three mAbs to whole cells of *E. coli* strains O18:K1 and O18:K⁻, cultured under various growth conditions are summarized in Table 1. To give an indication of the amount of antibody bound by bacteria from each growth condition, the A_{max} (maximum A_{590} reading obtained for the first dilution of the mAb) should be compared. The affinity which each antibody has for the bacteria from the various growth conditions is compared by calculating the D_{50} for each dilution curve, that is the dilution factor required to give the 50% value of the maximum absorbance reading. No significant differences in binding of the anti-O mAb to *E. coli* O18:K1 were detected for the different growth conditions. However, at high antibody dilutions (>1 in 160) the non-capsulate mutant *E. coli* O18:K⁻ showed a significant decrease in binding of mAb O-1 to cells grown under both iron and magnesium limitation (data not shown). On comparing the D_{50} values, the O18 non-capsulate strain showed stronger affinity for both anti-



core and the anti-O mAbs compared to its capsulate parent when grown under most growth conditions, the exceptions being the magnesium-depleted medium for the anti-O mAb and the iron-depleted medium for mAb C-2. Growth of both *E. coli* strains in the nitrogen-deficient/glucose-enriched medium resulted in much lower binding of the two anti-core mAbs compared to growth in nutrient broth. However, significant increases in A_{590} were observed when both strains were grown in serum and magnesium limitation and probed with mAbs

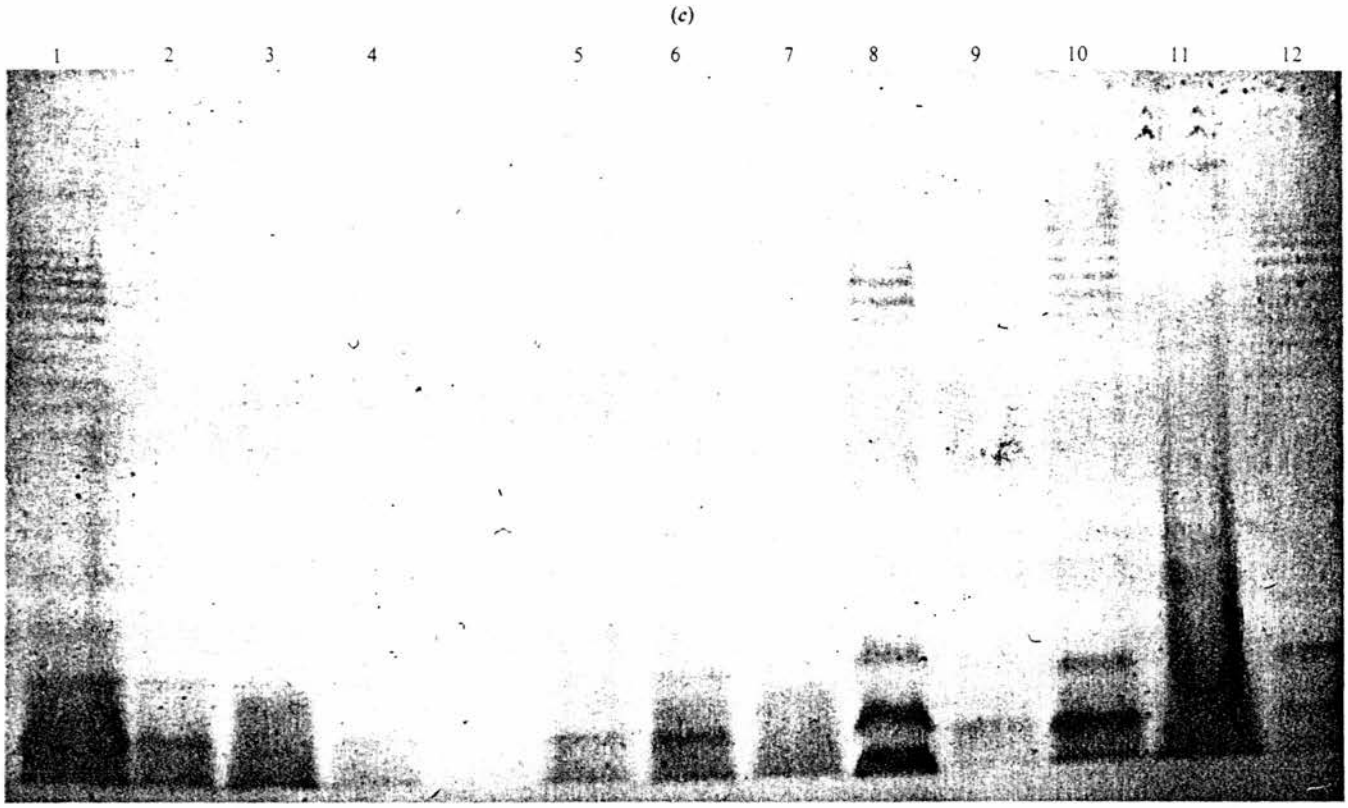


Fig. 1 (*a*) Silver-stained LPS profiles of proteinase K whole cell digests of four *E. coli* strains (O18:K1, O18:K⁻, O18:Krf and O6:K5) separated by PAGE (14%, w/v, acrylamide). (*b,c*) Immunoblots of the four *E. coli* strains transferred to nitrocellulose paper and probed with (*b*) an anti-O18 specific mAb, O-1, and (*c*) a core-specific mAb, C-2. Tracks 1–4, 5–8 and 9–12 represent the four *E. coli* strains grown to early stationary phase in nutrient broth, an iron-depleted medium and a nitrogen-deficient medium respectively.

Table 2. *Flow cytometric analysis of anti-LPS mAb binding to whole cells of four E. coli strains grown under different conditions.*

Percentage values represent the mean positive fluorescence of bacteria above background levels from three separate experiments. ND, Not done.

			Percentage of cells (mean ± SD) showing positive fluorescence			
mAb	Growth medium	*E. coli* . . .	O18:K1	O18:K⁻	O18:Krf	O6:K5
O-1	Nutrient broth		70(±19)	62(±14)	2(±1)	ND
	Nitrogen-deficient/ high-carbon		73(±10)	68(±7)	13(±6)	ND
	Iron-depleted		74(±6)	50(±3)	3(±1)	ND
	Serum		68(±15)	67(±8)	8(±1)	ND
	Magnesium-depleted		49(±9)	39(±9)	7(±0)	ND
C-1	Nutrient broth		3(±1)	3(±2)	68(±11)	7(±1)
	Nitrogen-deficient/ high-carbon		2(±0)	2(±1)	56(±12)	1(±0)
	Iron-depleted		10(±7)	7(±4)	66(±7)	7(±3)
	Serum		28(±16)	61(±11)	43(±14)	26(±12)
	Magnesium-depleted		21(±4)	24(±5)	75(±13)	18(±8)
C-2	Nutrient broth		2(±1)	6(±1)	26(±11)	3(±2)
	Nitrogen-deficient/ high-carbon		2(±0)	3(±1)	16(±6)	2(±1)
	Iron-depleted		3(±2)	8(±3)	20(±5)	14(±9)
	Serum		16(±5)	34(±11)	20(±12)	32(±8)
	Magnesium-depleted		12(±4)	18(±2)	28(±7)	10(±2)

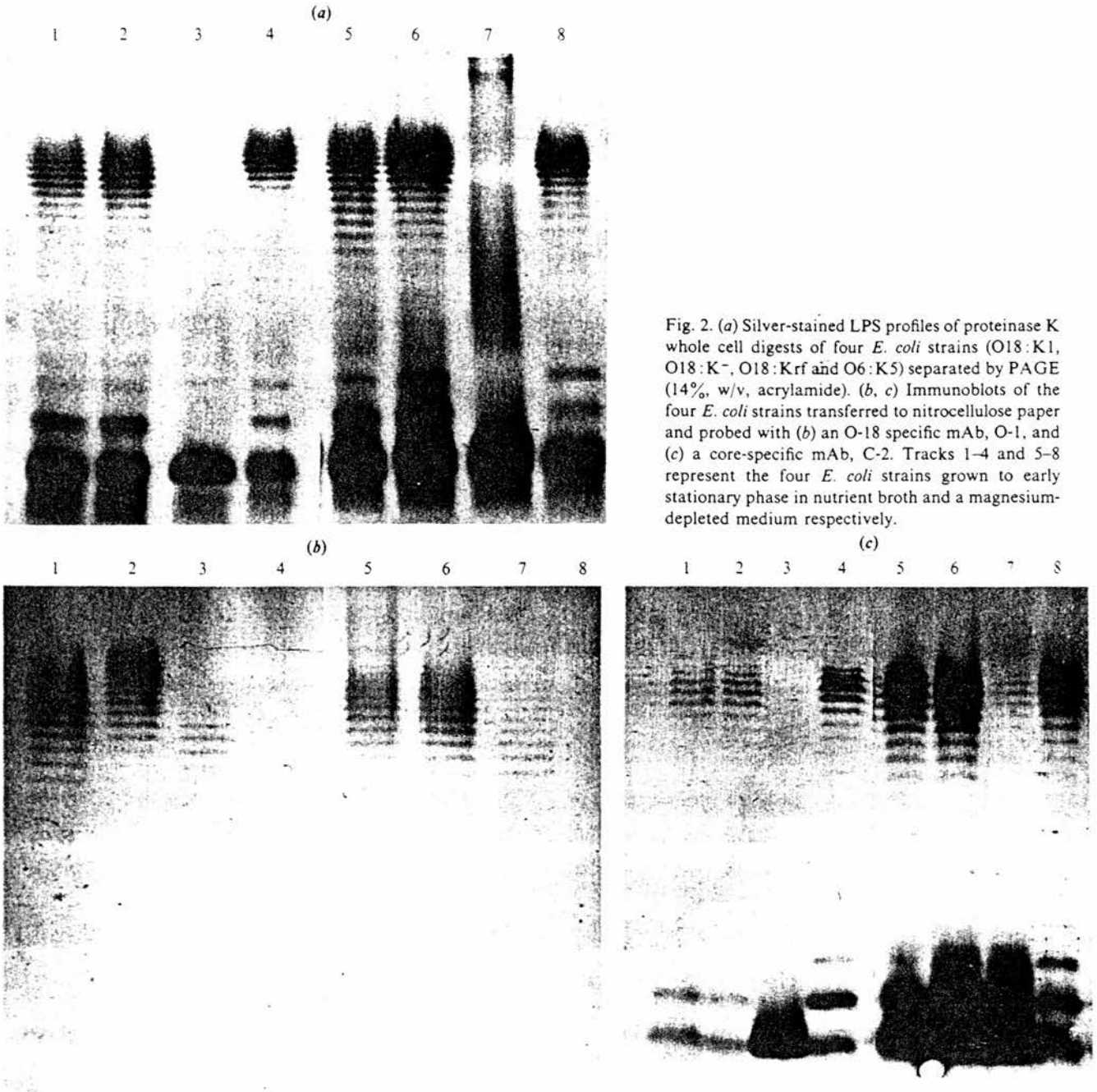


Fig. 2. (*a*) Silver-stained LPS profiles of proteinase K whole cell digests of four *E. coli* strains (O18:K1, O18:K⁻, O18:Krf and O6:K5) separated by PAGE (14%, w/v, acrylamide). (*b*, *c*) Immunoblots of the four *E. coli* strains transferred to nitrocellulose paper and probed with (*b*) an O-18 specific mAb, O-1, and (*c*) a core-specific mAb, C-2. Tracks 1–4 and 5–8 represent the four *E. coli* strains grown to early stationary phase in nutrient broth and a magnesium-depleted medium respectively.

C-1 and C-2. A similar, yet slightly lower increase in binding of anti-core mAbs was seen for growth under iron-limitation compared to nutrient broth.

Flow cytometric analysis

The effect of growth conditions on the expression of LPS on whole bacteria was also investigated by flow cytometry. Flow cytometric profiles, which relate to the intensity of fluorescence signal (i.e. antibody binding to bacteria), were obtained consistently in three separate experiments. Examples of representative profiles are illustrated in Fig. 4(*a, b*). Histograms representing interaction of the O18-specific mAb, C-1 and cells grown in nutrient broth and magnesium-depleted medium are shown in Fig. 4(*a*). A 41% decrease in magnesium-depleted bacteria exhibiting positive fluoresence above background levels compared to nutrient broth cells was

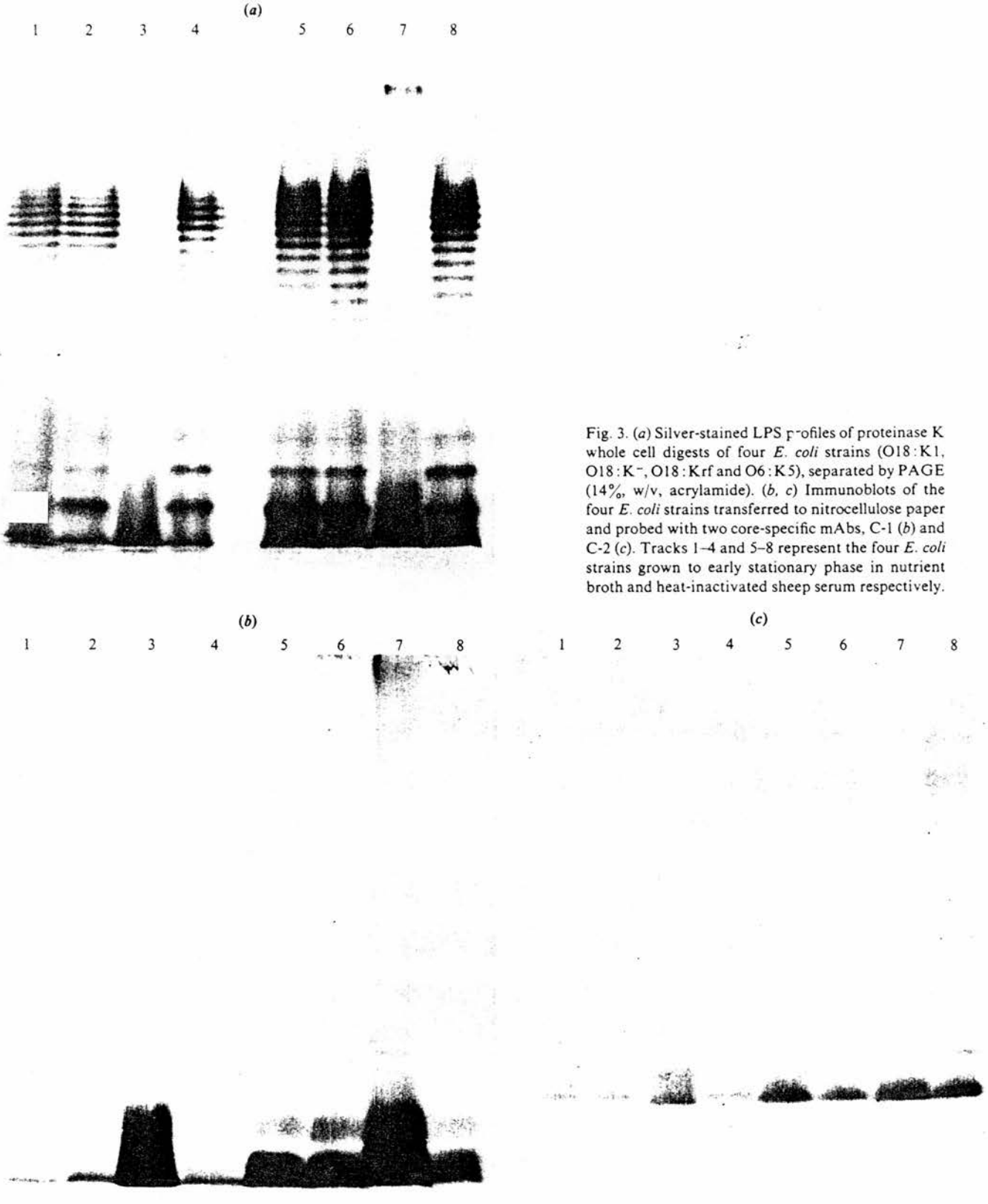


Fig. 3. (*a*) Silver-stained LPS profiles of proteinase K whole cell digests of four *E. coli* strains (O18:K1, O18:K$^-$, O18:Krf and O6:K5), separated by PAGE (14%, w/v, acrylamide). (*b*, *c*) Immunoblots of the four *E. coli* strains transferred to nitrocellulose paper and probed with two core-specific mAbs, C-1 (*b*) and C-2 (*c*). Tracks 1–4 and 5–8 represent the four *E. coli* strains grown to early stationary phase in nutrient broth and heat-inactivated sheep serum respectively.

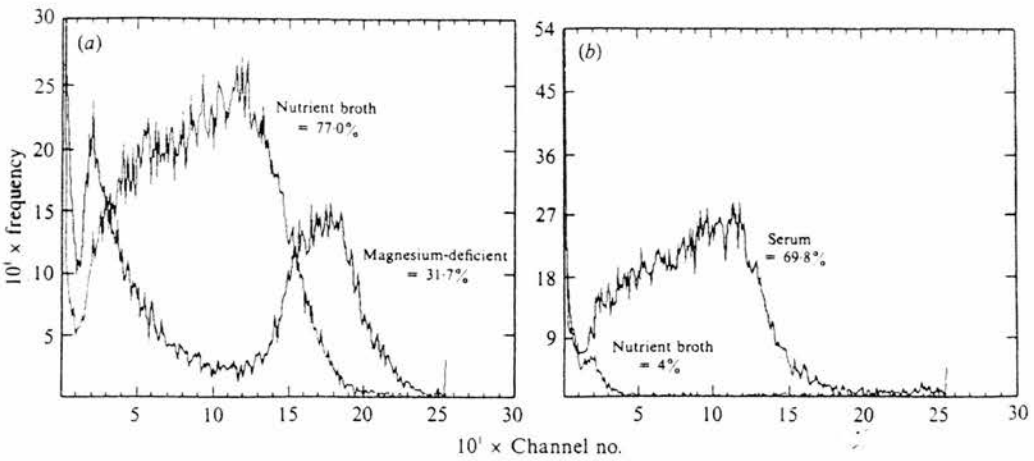


Fig. 4. Green fluorescence intensity histograms of (*a*) the anti-O18 mAb (O-1) against whole cells of *E. coli* O18:K1 grown to early stationary phase in nutrient broth and in a magnesium-depleted medium and (*b*) an anti-core mAb (C-1) against *E. coli* O18:K⁻ grown in nutrient broth and in a heat-inactivated sheep serum medium. Percentage values represent bacteria exhibiting positive fluorescence above background levels.

observed. The biphasic fluoresence pattern produced by magnesium-depleted cells demonstrated the presence of two distinct subpopulations of *E. coli* O18:K1 on the basis of differential binding by mAb O-1. Probing the same cells with C-1, a mAb reactive against unsubstituted core LPS, resulted in positive fluorescence levels of ~5% for nutrient broth and 29% for magnesium depletion. Significant increases in the binding levels of the two anti-core mAbs C-1 and C-2 (a mAb reactive against both substituted and unsubstituted core LPS) were also observed when *E. coli* O18:K⁻ cells were grown in serum compared to nutrient broth. The results obtained with C-1 are shown in Fig. 4(*b*), where serum-grown bacteria showed a positive fluorescence level 66% higher than nutrient-broth-grown bacteria.

Flow cytometry data, showing the effect of growing all four *E. coli* strains under the different growth conditions on mAb recognition of LPS-associated epitopes is presented in Table 2. Although relative differences between growth conditions remained constant, day-to-day variation in percentage labelling within a given sample was evident. Smooth cells grown with serum and under magnesium-depleted conditions again showed the largest increases in binding of anti-core mAbs. Iron-depleted and nitrogen deficient cells showed only minor differences compared to nutrient-broth-grown cells. Probing cells with the O-antigen-specific mAb resulted in similar binding levels for most conditions, whilst magnesium-depleted cells, notably O18:K⁻ showed a significant decrease. Enhanced binding of the two anti-core mAbs, especially C-1, was observed against the rough mutant O18:Krf, compared to the smooth strains. Minor variations between growth conditions of O18:Krf cells reacted with anti-core mAbs, favoured higher percentage labelling for bacteria grown in magnesium-depleted conditions.

Discussion

The environmental modulation of cell surface components of both Gram-positive and Gram-negative bacteria has been intensively investigated (Brown & Williams, 1985). Among the environmental factors that commonly influence the properties of microbial cells, the availability of essential nutrients assumes particular importance (Harder & Dijkhuizen, 1983). LPS is a major cell surface component of all Gram-negative bacteria, and is implicated as a cause of endotoxic shock (Ryan, 1985). This study has examined using mAbs, the effects of a number of nutrient growth conditions, relating to those *in vivo*, on the accessibility and expression of LPS antigens.

In an earlier study (Nelson *et al.*, 1990) we showed that immunoblotting, ELISA and flow cytometry could be used in combination to give both qualitative and quantitative data on how mAbs bind to LPS. Immunoblotting gives information as to the location of the epitope within the LPS molecule: viz. O-antigen, or substituted or unsubstituted core. ELISA with whole cells gives an extremely sensitive and quantitative indication of the binding of mAbs to whole cells, but suffers from the disadvantage that the procedure for immobilizing the bacteria to plastic may have distorting influences on the cells which may change binding characteristics. Flow cytometry lacks the sensitivity and

automation of ELISA but is quantitative and the binding occurs in physiological conditions.

The results presented in the current study indicate that the expression of *E. coli* core LPS increases when grown in magnesium-depleted conditions (0·17 mmol l^{-1}, cf. 0·65–1·0 mmol l^{-1} in serum). This was reflected in greater binding of anti-core mAbs in whole cell ELISA and flow cytometry, and this was supported by PAGE and immunoblotting. Magnesium is important in maintaining the stability of the structural arrangement of larger molecules such as LPS within the outer membrane of Gram-negative bacteria (Costerton *et al.*, 1974). Cell walls of magnesium-limited cells of *Bacillus subtilis* show an increased Mg^{2+}-binding affinity over magnesium-sufficient cells (Meers & Tempest, 1968). Thus, certain organisms may respond to magnesium limitation by improving their ability to bind the ions by increasing negatively charged cell surface components such as LPS. Using both batch and continuous culture Day & Marceau-Day (1982), also reported compositional changes in *Pseudomonas aeruginosa* LPS in response to Mg^{2+} concentration, reflecting functional alterations in the LPS. Data indicated a change in core size of LPS relative to the O-antigen component, suggesting the possibility of magnesium having a regulatory role on one or more of the LPS biosynthetic enzymes. An increased production of low molecular mass LPS was seen for *E. coli* grown at intermediate growth rates under magnesium limitation (Dodds *et al.*, 1987). The amino-sugar, 2-aminohexose was also found to be absent from the O-polysaccharide. Flow cytometric analysis of *E. coli* O18:K1 grown under magnesium-depleted conditions and probed with an O-antigen specific mAb revealed two populations of bacteria. Possible reasons for this include: differences in the structure or amount of LPS; the amount of capsule [shown to be inhibited under these conditions by Taylor *et al.* (1981)]; or morphological heterogeneity of bacteria grown under magnesium-depletion. The composition of LPS from *E. coli* O18:K^- LPS was shown to differ from nutrient-broth-grown LPS using nuclear magnetic resonance spectrometry (unpublished data). As well as a decrease in the amount of O-polysaccharide, rhamnose, a constituent of the O18 O-antigen, was found to be replaced by an as yet unidentified sugar.

The low availability of iron is acknowledged as a key determinant of virulence (Griffiths, 1987). Alterations in the outer membrane of many bacterial species under conditions of iron-deprivation include the production of high molecular mass, iron-regulated outer-membrane proteins (Neilands, 1982). Little effect of iron depletion on LPS production was observed by silver-staining and immunoblotting. However, increasing the degree of iron deprivation led to a greater expression of rough core at the expense of high molecular mass O-polysaccharide LPS (data not shown). Indeed, binding of anti-core mAbs to whole cells was shown to improve by whole cell ELISA (Table 1), although not significantly using flow cytometry (Table 2). This may be partly explained by differences in the two techniques for, as discussed above, ELISA is prone to distortions not present in flow cytometry, possibly leading to improved accessibility of antibodies to LPS epitopes. Although changes in the expression of LPS *per se* have been illustrated, growth under stress conditions such as iron or magnesium deficiency likely to alter the structure and composition of other components of the outer membrane. Therefore, LPS core determinants normally showing limited accessibility may become better exposed on the cell surface. Although this study attempted to assess the binding of anti-LPS mAbs to whole cells, release of membrane fragments from cells must also be considered. Indeed, variations in growth conditions could alter the extent and nature of the excretion of such fragments (Hoekstra *et al.*, 1976).

The present findings also show a significant increase in binding of anti-core mAbs when smooth *E. coli*, especially the non-capsulate O18:K^-, were grown in heat-inactivated sheep serum. Immunoblotting, ELISA and flow cytometry all showed marked increases in binding of mAbs to the core region, despite an apparent increase in the expression of O-antigen as revealed by silver staining. The possible presence of endogenous anti-LPS antibodies within the serum may have caused a false impression of mAb binding. However, since mAbs were raised in a species different from that in which the serum was obtained, antibody conjugates might be expected to recognize only mouse mAbs. Also, removal of any anti-LPS antibodies from the serum by absorption with bacteria had no effect on the results (data not shown). Thus heat-inactivated sheep serum appears to alter the antigenic expression of LPS to permit binding of mAbs to the core region. Chedid *et al.* (1968) proposed an enzymic process within serum capable of attacking cell wall components, thereby unmasking the conserved rough antigenic structures.

Although capsule plays an important role in the serum resistance of many bacteria (Leying *et al.*, 1990), it has been reported not to provide a barrier function for binding of anti-O antibodies to *Klebsiella* (Williams *et al.*, 1988) and some *E. coli* strains including O18 (Cross *et al.*, 1986). Indeed, binding of the anti-O18 mAb to capsulate and non-capsulate strains of *E. coli* O18 showed no effect due to the presence of capsule. However, greater binding of anti-core mAbs to the non-capsulate strain indicates capsules may have a function as a barrier for the penetration of anti-core LPS antibodies. Growth under nitrogen-deficient/high-car-

bon conditions has been shown previously to promote capsule formation (Sutherland & Wilkinson, 1965). Reduced binding of anti-core mAbs to both capsulate and non-capsulate bacteria grown under these conditions suggested another factor, other than capsule exerting an influence. Indeed, an observed increase in chain length and production of O-polysaccharide was revealed by silver staining and immunoblotting (Fig. 1). Since O-polysaccharide is known to reduce accessibility to core LPS (Gigliotti & Shenep, 1985), a change in both its production and possible arrangement may have strengthened this effect. A variety of growth conditions have been shown to alter the expression of O-antigenic LPS molecules. McGroarty & Rivera (1990) demonstrated a very marked decrease in the length of the O-specific LPS of cells grown under a number of stress conditions, whilst growth phase (Day & Marceau-Day, 1982) and growth rate (Dodds *et al.*, 1987) also influence its expression. Such variability in the expression of LPS polysaccharide formation may contribute towards the ability of bacteria to adapt to changes in the environment. Since variables other than nutrient limitation influence the expression of LPS, it is acknowledged that in using the batch culture model, the significance of altering one variable cannot be divorced with certainty from the influence of others.

We are grateful to Robin Barclay and colleagues at the S.E. Scotland Blood Transfusion Service for their on-going interest and collaboration in the project and their help in the selection of the monoclonal antibodies. Keith James and colleagues of the Surgery Department, University of Edinburgh are thanked for performing the fusions and producing the mAb supernates. We thank Bill Neill of the Department of Medical Microbiology for tirelessly and skillfully operating the flow cytometer. The work described here was funded by Sandoz, Basel, Switzerland and we thank Franco di Padova and Max Schreier for their help and encouragement.

References

AYDINTUNG, M. K., INZANA, T. J., LETONJA, T., DAVIS, W. C. & CORBEIL, L. B. (1989). Cross-reactivity of monoclonal antibodies to *Escherichia coli* J5 with heterologous Gram negative bacteria and extracted lipopolysaccharides. *Journal of Infectious Diseases* **160**, 846–857.

BROWN, M. R. W. & WILLIAMS, P. (1985). The influence of environment on envelope properties affecting survival of bacteria in infections. *Annual Review of Microbiology* **39**, 527–556.

CHEDID, L., PARANT, M., PARANT, F. & BOYER, F. (1968). A proposed mechanism for natural immunity to enterobacterial pathogens. *Journal of Immunology* **100**, 292–301.

CHIA, S. K. S., POLLACK, M., GUELDE, G., KOLES, N. L., MILLER, M. & EVANS, M. E. (1989). Lipopolysaccharide(LPS)-reactive monoclonal antibodies fail to inhibit LPS-induced tumor necrosis factor secretion by mouse-derived macrophages. *Journal of Infectious Diseases* **159**, 872–880.

COSTERTON, J. W., INGRAM, J. M. & CHANG, K. J. (1974). Structure and function of the cell envelope of Gram-negative bacteria. *Bacteriological Reviews* **38**, 87–110.

CROSS, A. S., KIM., K. S., WRIGHT, D. C., SADOFF, J. C. & GEMSKI, P. (1986). Role of lipololysaccharide and capsule in serum resistance of bacteremic strains of *Escherichia coli*. *Journal of Infectious Diseases* **154**, 497–503.

DAY, D. F. & MARCEAU-DAY, M. L. (1982). Lipopolysaccharide variability in *Pseudomonas aeruginosa*. *Current Microbiology* **7**, 93–98.

DODDS, K. L., PERRY, M. M. & MCDONALD, I. J. (1987). Alterations in the lipopolysaccharide produced by chemostat-grown *Escherichia coli* O157:H7 as a function of growth rate and growth-limiting nutrient. *Canadian Journal of Microbiology* **33**, 452–458.

DUNN, D. L., EWALD, D. C., CHANDAN, N. & CERRA, F. B. (1986). Immunotherapy of Gram negative bacterial sepsis. *Archives of Surgery* **121**, 58–62.

GIGLIOTTI, F. & SHENEP, J. L. (1985). Failure of monoclonal antibodies to core glycolipid intact smooth strains of *Escherichia coli*. *Journal of Infectious Diseases* **151**, 1005–1011.

GRIFFITHS, E. (1987). The iron uptake systems of pathogenic bacteria. In *Iron and Infection – Molecular, Physiological and Clinical Aspects*, pp. 69–138. Edited by J. J. Bullen & E. Griffiths. Chichester: John Wiley.

HANCOCK, I. C. & I. R. POXTON (1988). *Bacterial Cell Surface Techniques*. Chichester: John Wiley.

HARDER, W. & DIJKHUIZEN, L. (1983). Physiological responses to nutrient limitation. *Annual Review of Microbiology* **37**, 1–23.

HEUMANN, D., BAUMGARTNER, J. D., JACOT-GUILLARMOD, H. & GLAUSER, M. P. (1991). Antibodies to core lipopolysaccharide determinants: absence of cross-reactivity with heterologous lipopolysaccharide. *Journal of Infectious Diseases* **163**, 762–768.

HITCHCOCK, P. J. & BROWN, T. M. (1983). Morphological heterogeneity among *Salmonella lipopolysaccharide* chemotypes in silver-stained polyacrylamide gels. *Journal of Bacteriology* **154**, 269–277.

HOEKSTRA, D., VAN DER LAAN, J. W., DE LEIJ, L. & WIT, B. (1976). Release of outer membrane fragments from normally growing *Escherichia coli*. *Biochemica et Biophysica Acta* **455**, 889–899.

DE JONGH-LEUVENINK, J., SCHELLEKENS, J. & VERHOEF, J. (1990). Characterization of anti-core glycolipid monoclonal antibodies with chemically defined lipopolysaccharides. *Infection and Immunity* **58**, 421–426.

KELLY, N. M., BELL, A. & HANCOCK, R. E. W. (1989). Surface characteristics of *Pseudomonas aeruginosa* grown in a chamber implant model in mice and rats. *Infection and Immunity* **57**, 344–350.

KIPPS, T. J. & L. A. HERTZENBERG (1986). Schemata for production of monoclonal antibody-producing hybridomas. In *Handbook of Experimental Immunology*, pp. 108.1–108.9. Edited by D. M. Weir. Oxford: Blackwell.

LAEMMLI, U. K. (1970). Cleavage of structural proteins during assembly of the head protein of bacteriophage T4. *Nature, London* **227**, 680–685.

LEYING, H., SUERBAUM, S., KROLL, H-P., STAHL, D. & OPFERKUCH, W. (1990). The capsular polysaccharide is a major determinant of serum resistance in K-1-positive blood culture isolates of *Escherichia coli*. *Infection and Immunity* **58**, 222–227.

MCGROARTY, E. J. & RIVERA, M. (1990). Growth-dependent alteration in production of serotype-specific and common antigen lipopolysaccharides in *Pseudomonas aeuroginosa* PAO1. *Infection and Immunity* **57**, 1030–1037.

MAYORAL, J. L. & DUNN, D. L. (1990). Cross-reactive murine monoclonal antibodies directed against the core/lipid A region of endotoxin inhibit production of tumor necrosis factor. *Journal of Surgical Research* **49**, 287–292.

MEERS, J. L. & TEMPEST, D. W. (1968). The influence of extracellular products on the behaviour of mixed microbial populations in magnesium-limited chemostat cultures. *Journal of General Microbiology* **52**, 309–317.

MORSE, S. A., MINTZ, C. S., SANFIA, S. K., BAUERTENSTEIN, L., BORTRAM, M. & ARCELLA, M. A. (1983). Effect of dilution rate on lipopolysaccharide and serum resistance of *Neisseriae gonorrhoeae* grown in continuous culture. *Infection and Immunity* **41**, 74–82.

NEILANDS, J. B. (1982). Microbial envelope proteins related to iron. *Annual Review of Microbiology* **36**, 285–309.

NELSON, D., NEILL, W. & POXTON, I. R. (1990). A comparison of immunoblotting, flow cytometry and ELISA to monitor the binding of anti-lipopolysaccharide monoclonal antibodies. *Journal of Immunological Methods* **133**, 227–233.

POLLACK, M., CHIA, J. K. S., KOLES, N. L., MILLER, M. & GUELDE, G. (1989). Specificity and cross-reactivity of monoclonal antibodies reactive with core and lipid A regions of bacterial lipololysaccharide. *Journal of Infectious Diseases* **159**, 168–184.

ROBERT-GERO, M., POIRET, M. & COHEN, G. N. (1970). The aspartate kinase of *Pseudomonas putida*, regulation of synthesis and activity. *Biochemica et Biophysica Acta* **206**, 17–30.

RYAN, J. L. (1985). Microbial factors in pathogenesis: lipopolysaccharides. In *Septic Shock (Contemporary Issues in Infectious Diseases)*, pp. 13–25. Edited by K. Root & M. A. Sande. New York: Churchill Livingstone.

SCOTT, B. B. & BARCLAY, G. R. (1987). Endotoxin–polymyxin complexes in an improved enzyme-linked immunosorbent assay for IgG antibodies in blood-donor sera to Gram-negative endotoxin core glycolipids. *Vox Sanguinis* **52**, 272–280.

SMITH, A. W., WILTON, J., CLARK, S. A., ALPAR, O., MELLING, J. & BROWN, M. R. W. (1991). Production and characterization of monoclonal antibodies to outer membrane proteins of *Pseudomonas aeruginosa* grown in iron depleted media. *Journal of General Microbiology* **137**, 227–236.

SUTHERLAND, I. W. & WILKINSON, J. F. (1965). Depolymerases for bacterial exopolysaccharides obtained from phage-infected bacteria. *Journal of General Microbiology* **39**, 373–383.

TAYLOR, P. W., MESSNER, P. & PARTON, R. (1981). Effect of the growth environment on cell-envelope components of *Escherichia coli* in relation to sensitivity to human serum. *Journal of Medical Microbiology* **14**, 9–19.

TERRY, J. M., PINA, S. E. & MATTINGLY, S. J. (1991). Environmental conditions which influence mucoid conversion in *Pseudomonas aeruginosa* PAO1. *Infection and Immunity* **59**, 471–477.

TOWBIN, H., STAEHELIN, T. & GORDON, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proceedings of the National Academy of Sciences of the United States of America* **76**, 4350–4354.

TSAI, C.-M. & FRASCH, C. E. (1982). A sensitive silver stain for detecting lipopolysaccharide in polyacrylamide gels. *Analytical Biochemistry* **119**, 115–119.

WILLIAMS, P., LAMBERT, P. A. & BROWN, M. R. W. (1988). Penetration of immunoglobulins through the *Klebsiella* capsule and their effect on cell-surface hydrophobicity. *Journal of Medical Microbiology* **26**, 29–35.

ZIEGLER, E. J., FISHER, C. J., SPRUNG, C. L., STRAUBE, R. C., SADOFF, J. C., FOULKE, G. E. & WORTEL, C. H. (1991). Treatment of Gram-negative bacteremia and septic shock with HA-1A human monoclonal antibody against endotoxin. *New England Journal of Medicine* **324**, 429–436.